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National Institute of Arthritis, Metabolism, and Digestive Diseases.
Report of program activities

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ANNUAL PROJECT REPORTS

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ANNUAL REPORT SUMMARY
LABORATORY OF NUTRITION AND ENDOCRINOLOGY
NATIONAL INSTITUTE OF ARTHRITIS, METABOLISM & DIGESTIVE DISEASE

## MEMBRANE REGULATION SECTION

### Mechanism of Action of Hormones and Guanine Nucleotides on Adenylate Cyclase Systems

Modelling of the transient kinetics of guanine nucleotide activation of adenylate cyclase in hepatic membranes has been carried out with the SAAM program developed by Dr. Mones Berman. The model developed extends the previously reported three state model and provides an explanation for the differences both in effectiveness and transient kinetics observed with Gpp(NH)p and GTP. In essence, the model suggests that, in addition to the catalytic site that converts ATP to cyclic AMP, the enzyme system contains a GTPase at the nucleotide regulatory site that converts GTP to inactive product, GDP. One important outgrowth of this model is that it serves to explain the rapidly reversible dynamic features of hormone and GTP activation of adenylate cyclase. It also serves to explain the actions of cholera toxin on adenylate cyclase, as related below.

### Studies of Binding of [$^{3}$H]glucagon to Receptors in Hepatic Membranes

Studies with [$^{3}$H]glucagon have helped to resolve some important questions arising from previous studies on the binding of [$^{125}$I]glucagon to receptors in hepatic membranes; [$^{3}$H]glucagon, in contrast to [$^{125}$I]glucagon, binds and activates adenylate cyclase with the same kinetic characteristics obtained with native glucagon provided that GTP is present in the medium. Under these conditions, it was found GTP promotes the formation of two states of the glucagon receptor. One state, comprising about 10% of the total, has a high affinity for glucagon, shows positive cooperative binding (Hill coefficient >2) and has the essential kinetic features of the receptor that is coupled to adenylate cyclase. The second state formed by GTP has a low affinity for glucagon ($K_D$ > 10 nM) and is not coupled to adenylate cyclase. We suggest from these findings that the glucagon receptor is present in about 10-fold excess over that of adenylate cyclase in hepatic membranes. GTP converts the receptor from an essentially irreversible binding state that is uncoupled to adenylate cyclase to a state that is compatible for "coupling" with adenylate cyclase, the amount coupled being limited by the concentration of adenylate cyclase, hormone, and GTP.

### Structure Function Relationships for Glucagon

We are continuing efforts to modify various residues in the glucagon molecule in order to prepare derivatives that may be useful as competitive antagonists, for affinity tagging of the glucagon receptor, and for determining structure-function relationships. The lysine residue at position 12 has hampered our previous efforts to specifically modify the alpha amino group of the N-terminal histidine residue. We have now succeeded in converting, with high

yield, the lysine residue to homoarginine with full retention of the biological activity of the resultant homoarg$^{12}$-glucagon. The amino terminal residue has been removed by treatment with phenyl isothiocyanate treatment; the resultant desHis$^{12}$, HomoArg$^{12}$-glucagon is, like DesHis$^{1}$ -glucagon, a partial agonist on hepatic adenylate cyclase. Preliminary studies indicate that incorporation of N-acetyl homocysteine in place of histidine regenerated substantial activity previously lost due to removal of the His$^{1}$-residue from glucagon. This finding suggests that while the amino terminal residue of glucagon is important to its activity, histidine itself does not play an essential role.

Studies on Hormone Responses in Cultured Cells.

Last year we initiated a long-range program of study on cultured hepatocytes with the aim of understanding the primary actions of growth hormone and other hormones such as insulin, catecholamines, and glucagon on cultured cells. We have succeeded in showing that responsiveness of glucagon and epinephrine (on adenylate cyclase or cyclic AMP production) can be preserved for more than one month in primary and secondary cultures of fetal rat liver. However, certain culture conditions result in loss of glucagon response. We are currently investigating whether this loss is related to loss of glucagon receptors or possibly of the guanine nucleotide regulatory components associated with enzyme and receptor.

Regulation of Adenylate Cyclase by Adenosine

We reported last year that adenosine interacts with a specific "receptor" site on the hepatic adenylate cyclase system that results in marked inhibition of enzyme activity. The $K_i$ for adenosine was decreased at least 50-fold by the actions of glucagon and divalent cations, indicating that the latter ligands regulate the actions of adenosine at its receptor site. These studies have been followed up, in collaboration with Dr. Jan Wolff, by investigating the actions of adenosine on adenylate cyclase in membranes from several cell types. Very interesting findings were obtained by using a variety of analogs of adenosine which are modified in the purine and/or the ribose moieties of the molecule. It has been found that the effects of adenosine are mediated by at least two markedly different receptors which show preferential binding with analogs that are modified either at the ribose or purine moieties. Those that are modified at the ribose moiety interact selectively with the adenosine receptor that causes inhibition of cyclase activity. By contrast adenosine analogs with alterations in the purine moiety react selectively with an adenosine receptor that causes activation of adenylate cyclase. In liver cells, only the inhibitory adenosine receptor is detected. In Leydig cells, only the stimulatory adenosine receptor is present. However, in some cells, such as human blood platelets, both receptors are present, resulting in biphasic stimulatory and inhibitory effects of adenylate cyclase activity depending on the concentrations of adenosine or the type of adenosine analog added to the medium. These findings may have broad physiological significance since actions of adenosine on cells have been known for many years without a coherent pattern which suggests mechanism. We are now following up the actions of adenosine on both cyclic AMP production in intact cells as well as on

adenylate cyclase activity in broken cell preparations. Since the adenosine analogs show specificity toward the inhibitory and stimulatory forms of the adenosine receptor, it should be possible to evaluate the type of receptor present and to determine whether cyclic AMP production is the primary reflectant of the actions of adenosine in biological systems.

Purification and Characteristics of the Hepatic Adenylate Cyclase System

The following was achieved during the past year:

1) The glucagon receptor complex has been separated from adenylate cyclase after solubilization of the enzyme system with Lubrol PX. Both the isolated glucagon-receptor complex and the enzyme are affected by guanine nucleotides in the same manner observed with receptor and enzyme in intact membranes. These studies suggest that the hormone receptor and the enzyme are each linked to a guanine nucleotide regulatory site or component.

2) It was estimated from the quantity of [$^3$H]Gpp(NH)p binding sites associated with partially purified adenylate cyclase that at most 0.5 to 0.7 pmol of guanine nucleotide sites can be associated with adenylate cyclase in the membrane. This value contrasts with about 5 pmol glucagon receptor sites in the membranes; i.e., about 10 times that of the enzyme. This agrees with our [$^3$H]glucagon binding studies which suggest that, of the total receptors in the membranes, about 10% are coupled to adenylate cyclase in the presence of GTP. It appears, therefore, that there is a "receptor pool" for glucagon in hepatic membranes. We speculate that this pool of receptors is the source of the precursor states of the receptor that, when occupied by GTP at a site linked to the receptor (see below) is converted to a form compatible for "coupling" to adenylate cyclase and formation of the hormone-sensitive, coupled form of the enzyme system. In the absence of GTP, glucagon binding converts this pool to a form of the receptor that is not capable of interacting with adenylate cyclase and which may be the so-called "desensitized" receptor reported for other hormone-sensitive cyclase systems.

3) As noted above, glucagon receptor and enzyme are separate molecular entities and respond to guanine nucleotides when separated by gel chromatography. Guanine nucleotides convert the bulk of the receptors to an uncoupled, low affinity state of the receptor and the enzyme to a high activity state. We have evaluated the characteristics of the effects of guanine nucleotides on the receptor and the enzyme. Our findings indicate that the nucleotide sites or components linked to the receptor and enzyme have very different characteristics with respect to the affinities of various analogs of GTP for these sites, the effects of chelating agents, phospholipases, and the structural requirements for nucleotide action. We conclude from these findings that the nucleotide regulatory sites or components linked to receptor and enzyme are different. However, until these components can be isolated and characterized we cannot state whether these components represent different molecules. Much of our current efforts are being directed toward this goal.

4) As reported last year we encountered difficulties in our attempts to purify adenylate cyclase beyond the 4-to 5-fold purification achieved on molecular sieving columns. We believe that this difficulty stems from the formation of stable phospholipid-detergent aggregates of the enzyme along with other

proteins found in the partially purified fraction. Partly because of this, we have avoided further characterization of the partially purified enzyme. However, we have chosen two different techniques that might give some estimates of the molecular size of the enzyme and its possible conformation after subjecting the membrane-form of the enzyme to various activating ligands (fluoride ion, Gpp(NH)p, GTP+glucagon). Although still preliminary, some promising results have been obtained and are, therefore, reported here.

Using the linear accelerator at the Naval Medical Institute, we have irradiated hepatic membranes with varying doses of high energy electrons and measured the decay in adenylate cyclase activity as a function of the radiation dose. Such procedures have been used to evaluate the molecular size of many biological macromolecules and, in carefully controlled and conducted experiments, have given molecular sizes similar or even identical to those obtained with more conventional procedures. Our initial efforts are directed more toward determining whether the various activating ligands alter the apparent size distribution of adenylate cyclase rather than to an absolute molecular size of the functional unit of adenylate cyclase. The results obtained thus far indicate that the size of the enzyme system reflects the type of ligands used in activation. In the case of fluoride ion, the predominant size is about 400,000. However, glucagon and GTP act preferentially with a unit having a molecular size at least double that value; Gpp(NH)p also acts predominantly on the unit which is larger than that activated by fluoride ion. The glucagon receptor displays a single species of molecular weight in the range of 500,000.

The second procedure that we have evaluated is the PAGE system developed by Dr. Crumbach which involves the use of gel electrophoresis and the characteristics of protein migration as a function of gel porosity and the charged environment to which the proteins are exposed. By employing various types of buffered systems and gel porosities, we have found that Lubrol-solubilized adenylate cyclase takes on different configurations after previous activation of the membrane-bound enzyme with the various effector ligands. It would appear that the enzyme does not take the same configuration upon activation by the ligands but seems to reflect the type of ligand that brings about the activation. This agrees with the findings from the irradiation experiments and may be telling something about the nature of the components responsible for activation by fluoride ion, Gpp(NH)p, and glucagon.

Purification of Testicular Adenylate Cyclase

Two important goals of the section are to purify the adenylate cyclase solubilized from membranes and to quantify the number of cyclase molecules present in the membranes. As a possible means of accomplishing both objectives, we are attempting to purify a soluble form of adenylate cyclase present in the mature rat testis; though soluble, it is conceivable the primary structure of the soluble testis enzyme is similar to or even identical to the adenylate cyclase bound to membranes. Thus, antibodies to purified test enzyme may interact, for example, with the hepatic adenylate cyclase and thus allow, with appropriately established methods quantitation of the amount of hepatic enzyme, perhaps localize the enzyme in its membrane form, and purify, on antibody-

affinity columns, the enzyme after solubilization. We have succeeded in purifying the testis enzyme 500-fold with the use of $(NH)_4)_2SO_4$ precipitation, DEAE-argarose chromatography, Sephadex G-150 chromatography, ATP-agarose affinity columns. Hydroxy-apatite columns have recently given another 10-fold purification. Thus we are now in position to develop antibodies to the testis enzyme for the purposes outlined above.

Reversible Activation of Adenylate Cyclase by GTP analogs

$Gp(CH_2)pp$ activates hepatic adenylate cyclase in essentially the same manner as $Gpp(NH)\bar{p}$ or $Gpp(CH_2)p$; activation is slow in onset and leads to a high activity state that is slowly reversible. It was found that the terminal phosphate of $Gp(CH_2)pp$ is not hydrolyzed by GTPases in hepatic membranes possibly because the bond angles in the methylene bridge shift the terminal phosphate out of the sites responsible for catalytic breakdown. The significance of these findings is that they rule out the possibility that activation of adenylate cyclase systems by GTP and its analogs is through a covalent modification of the nucleotide regulatory site; $Gp(CH_2)pp$ cannot be utilized in pyrophosphate transferase reactions and is stable to nucleotide phosphohydrolase action. The findings also lend further indirect evidence for a GTPase at the site of activation of adenylate cyclase.

The Mechanism of Cholera Toxin Activation of Adenylate Cyclase.

The $A_1$ subunit of cholera toxin has been purified and shown to activate adenylate cyclase prepared from turkey erythrocytes. Activation of adenylate cyclase is completely dependent on GTP and NAD; the latter is required as substrate for the ADP-ribose transferase activity of the $A_1$ subunit. After pretreatment of adenylate cyclase with $A_1$ plus NAD, followed by washing the membranes, little adenylate cyclase activity is expressed unless GTP is present. The pretreated enzyme exposed to GTP shows the same persistent activated state previously reported with Gpp(NH)p. These findings suggest that cholera toxin $A_1$ produces a covalent modification of the nucleotide regulatory component involved in GTP action. The explanation for the persistent activation may be blockage of the GTPase that we believe to be present on the nucleotide regulatory component. Further studies are underway on the actions of $A_1$ on the hepatic enzyme system which is more difficult to evaluate because NAD causes inhibition of this system. When the cause of this effect of NAD is known we will proceed to detailed studies of the mechanism by which cholera toxin inhibits the GTPase associated with hepatic adenylate cyclase.

DEVELOPMENTAL BIOCHEMISTRY SECTION

Histone-DNA Interactions in the Chromatin Core Particle

Extensive investigations of various features of the structure of the chromatin core particle have been performed, using a variety of methodological approaches. Basic to a number of these studies is labeling isolated core particles at the 5' end of the DNA; using this modified particle, one can, by autoradiography of electrophoretic gels, determine not only the sizes of DNA fragments generated when the core particles are digested with various nucleases, but also the positions of cleavage sites and their relative susceptibilities to cleavage.

DNase I, DNase II and Aspergillus oryzae endonuclease cleave core particle DNA at the identical sites, evenly spaced at ten nucleotide intervals from the ends of the DNA. Those nucleases which require divalent cations fail to readily hydrolyze the sites 30, 60-80 and 110 bases from the 5' end. Micrococcal nuclease also does not hydrolyze the central region of core particle DNA; however, it makes its double stranded cuts at sites different from the regular ten nucleotide interval sites characteristic of digestion by the other enzymes.

We have investigated what features of core particle structure might lead to the nuclease resistant sites within the chromatin subunit. Removal of the highly basic amino terminal regions of the core particle histones by limited tryptic proteolysis generates a particle which still contains partially folded DNA. The pattern of nicking sites at ten nucleotide intervals from the ends persists in this derivative particle. Remarkably, however, the resistance to cleavage by DNase I of the central region of the DNA, 60-80 bases from the 5' ends, is lost in the trypsin treated subunit. Additionally, the 5'-terminal label is now very rapidly degraded by micrococcal nuclease, while a smaller increase in rate of digestion is detected for bulk core particle DNA. We thus operationally define the sites 0, 60-80, and 140 bases from the 5' ends of core particle DNA as binding sites for the basic amino terminal regions of the histones.

To determine which histones bind to DNA at the ends of the core particle, we have utilized a chemical crosslinking procedure initially described by Levina and Mirzabekov. The procedure crosslinks the 5'-terminal label to the histone which is its nearest neighbor in the particle. Initial results were complicated by the presence of a protein kinase in the core particles which phosphorylated histones H3, H2B, H4, as well as nonhistone proteins. Removal of the kinase by salt washing has allowed the putative identification of histone H3 as the protein which binds the nucleic acid at the points where it enters and exits from its path around the protein nucleus of the core particle.

Histones H3 and H4 appear to play a major role in folding and compaction of DNA in the core particle. The arginine rich histones alone compact 140 base pair DNA into a spherical particle morphologically similar to native core particles, with a sedimentation coefficient of 9.8 S. The partial particle contains a tetramer of the arginine rich histones. These histones can also organize the structure of larger pieces of DNA. Association of H3 and H4 with SV40 DNAI leads to formation of relaxed structures which contain about 17 spherical particles per molecule of DNA. The length compaction of this complex allows us to calculate that the arginine rich histones fold 160 base pairs of DNA into each particle, while all four smaller histones fold 170 base pairs of DNA. H3 and H4 also induce torsional constraints onto SV40 DNA. The extent of alteration in the topological properties of either DNAI or DNAIr is quite similar for equal molar ratios of either H3 and H4 alone, or when in combination with H2A and H2B. We conclude that these arginine rich histones, whose sequences have been highly conserved throughout evolution, organize the basic features of the structure of the core particle; this structure is then presumably refined by histones H2A and H2B.

Dimethyl suberimidate can be used to crosslink the histones of the core particle into an octameric complex. This single protein can then be isolated and shown to recombine with 140 base pair DNA to form a complex which is identical in a number of properties with its parent crosslinked particle. Both dissociation and reassociation take place in the absence of denaturants. The octamer also compacts and induces torsional constraints on SV40 DNA. Such studies convincingly show that DNA must be external to the protein core in the chromatin subunit and that energy derived from histone-histone interactions is not required in the kinetic mechanism for folding of DNA in chromatin.

## Immunochemical Studies of Chromatin Structure-function Relationships

Antibodies specific to the individual histones have been prepared, purified and then utilized to probe the presence and availability of histones in various systems differing in their functional activity. A problem in such studies is the degree of immunological relatedness of the immunogen and the antigen under study. Using quantitative complement fixation, we have been able to demonstrate a very high degree of crossreactivity for antibodies to calf histones H2A, H2B, H3 and H4 with histones derived from Drosophila tissue culture cells. This finding suggests that anti-calf antibodies can serve as universal reagents for the types of studies to be described.

Use of a new method for immunological studies, immunosedimentation, has allowed examination of the histone composition of individual chromatin core particles. Core particles are reacted with histone-specific antibodies and then the mixture of reacted and unreacted particles separated by sedimentation. The particles which have bound antibody sediment faster than those which have not. Using this approach, we have been able to show that every core particle contains two each of the four smaller histones. Thus, functional diversity in core particles is not reflected by molecular heterogeniety in their histone content.

Two systems which allow examination of the histone content of individual genomic units have been studied in collaboration with others. Antihistone antibodies clearly demonstrate the presence of these basic proteins in the spherical particles present on nonribosomal genes actively being transcribed in preblastoderm embryos of Drosophila melanogaster. A full complement of histones is also present in both daughter chromatids in newly replicated chromatin in the same organism. In the polytene chromosomes of Chironomous thummi, antihistone antibodies stain bands and interbands with intensities proportional to their contrast densities, suggesting a uniform relationship of histones to DNA throughout the chromosome. In contrast, puffed regions stain weakly or not at all with antihistone antibodies. Based on other results, we think that this is likely due to shielding of the antigenic determinants of the histones in puffs by nonhistone proteins, although the possibility of fundamental differences between polytene and monotene chromatin remains.

## Concanavalin A: A Probe for Chromosomal Glycoproteins

Concanavalin A binds specifically to liver chromatin with a dissociation constant of about $10^{-7}$ M. The association and dissociation kinetics indicate a preponderance of one type of binding sites; the Scatchard plot of equilibrium data is consistent with this view. Binding is inhibited by sugars that are known to interact with the lectin. Determination of the site of binding by staining protein gels with the lectin demonstrates that three protein bands constitute the primary sites of interaction on concanavalin A with chromatin. This molecule can be used to probe the structural and functional organization of a restricted group of nonhistone chromosomal proteins in a fashion exactly analogous to studies detailed previously with antihistone antibodies.

## Interactions of Triiodothyronine (T3) with Chromatin

Using a new binding assay for study of the interactions of T3 with chromatin, we have been able to show that a small number of binding sites (500-1000) bind the hormone with high affinity. The binding of hormone by these sites appears to be physiologically relevant: the sites are largely occupied in non-thyroidectomized rats. Binding of T3 to chromatin exhibits cooperative kinetics-both

Hill and Scatchard analysis of binding are consistent with a dimer of the hormone receptor being the minimal functional species.

## Preparation of Length and/or Sequence Defined DNA Oligomers

An armamentarium of length and/or sequence defined DNA oligomers is fundamental to studies of DNA-histone association in reconstituted core particles. Two approaches are being utilized to generate such a panel of DNA fragments. First, different enzymes have been isolated and their limit digest patterns determined using several DNA sources. Most promising is an endonuclease from Aspergillus oryzae which yields a group of oligomers with chain lengths varying from 10-200 on digestion of calf or E. coli DNA. This enzyme might be useful for preparation of very short oligomers of defined length although variable sequence.

A second approach has been to isolate and charaterize the cleavage products of a number of restriction endonucleases acting on bacteriophage DNA. A number of the enzymes have been isolated and optimal conditions for cleavage and subsequent purification of DNA fragments determined. The availability of defined length and sequence fragments of DNA should allow studies of the interactions of histones and DNA in reconstituted core particles at the molecular level, analogous to the types of studies so elegantly performed for the interactions of the lac repressor with the lac operator.

## Interactions of a Prokaryotic Ribonuclease with Its Inhibitor

The extracellular ribonuclease (barnase) of Bacillus amyloliquefaciens has been further studied in terms of features of its structure which lead to stability and enzymatic activity. Complementation of the protein lacking the C-terminal eight residues by a number of peptides demonstrates that an overlap of at least seven residues is necessary to lead to tight binding and restoration of activity. Iodination of barnase leads to retention of activity with substitution of Tyr-117 and possibly of either Tyr-24 or His-18. Other potentially susceptible residues are not modified. The modified barnase crystallizes in a morphology similar to that of the native molecule, suggesting that this modification might provide the heavy-atom isomorphous replacement needed to allow completion of the crystal structure of the protein.

## Study of a Functional Transition In a Haploid Organism

The haploid gametophyte generation of the mosses is marked by two distinct morphological stages; the filamentous protonema is followed by the mature gametophore. This provides an opportunity to study a major transition in gene activity in a haploid organism, increasing the chances of genetic studies markedly. The moss Nanomitrium austinii has been brought into aseptic culture and its development in culture studies. Either a decrease in temperature (25° to 15°) or application of very low concentrations of kinitin brings on the development of gametophores from the protonemal filiments. The transition induced by temperature shift is completely reversible prior to the stage of production of diploid sporophytes.

## VITAMIN METABOLISM SECTION

Chicken liver dihydrofolate reductase is shown to contain a single cysteine

residue and thus a single sulfhydryl group. This functional group is found to be readily titrated by both p-hydroxymercuribenzoate and dithionitrobenzoate although the dithionitrobenzoate reacts much more slowly than the organic mercurial. The binding of p-hydroxymercuribenzoate as well as a series of other organic mercurials such as methylmercuric hydroxide results in a marked stimulation of the reductase reaction. In contrast, the binding of thionitrobenzoate results in inhibition of activity. Evidence is presented that suggests that this sulfhydryl group is not at the active site of the enzyme since the interaction with the mercurials is not affected by the presence of a large excess of substrate. These results suggest that binding of a specific substance to this sulfhydryl group results in a conformational change in the protein yielding a more active enzymatic species. However attempts to demonstrate such a conformational change by a variety of methods have not been successful. Thus the effect on the tertiary structure of the protein must be very small. Kinetic studies suggest that the end result of this change may be related to the binding of the substrates.

## SECTION ON NUTRITIONAL BIOCHEMISTRY

### Vitamin E Nutrition and Metabolism

Further studies of the binding protein for α-tocopherol in rat liver showed it to be specific for d-α-tocopherol. The binding protein was found in liver of six other animal species but in no other tissue. α-Tocopherol bound to the protein was no more effective in preventing microsomal lipid peroxidation than was free tocopherol. In studies of the exchange of α-tocopherol between plasma and red cells, the distribution at equilibrium was markedly affected by the hematocrit value and also by the level of plasma total lipids.

### Biochemical Studies of Hepatic and Intestinal Function

Continuing studies of the metabolism of rat small intestine perfused *in situ* have shown that the major substrates utilized from blood (post-absorption) are glutamine (35%), 3-hydroxybutyrate (26%) and acetoacetate (24%). Much smaller oxidation of glucose, lactate and unesterified fatty acids occurred. End-products of the substrates were identified as ammonia, alanine, citrulline, proline, lactate, citrate, $CO_2$ and glucose. Lactate was found to be in rapid equilibrium between the blood and intestinal cells. These studies provide the first overall view of the intermediary metabolism of the small intestine under physiological conditions.

## SECTION ON ENDOCRINOLOGY

### Mechanism of Hormonal Regulation of Lipoprotein Lipase Activity in Mammary Gland

Lipoprotein lipase activity is increased in mamary gland and decreased in adipose tissue during lactation. Earlier studies showed that these changes in enzyme activity are mediated through prolactin secretion by the anterior pituitary gland. The present study was initiated to determine the mechanism of hormonal regulation of lipoprotein lipase activity in mammary gland cells.

During the past year we have developed a suitable primary mammary cell culture system with which hormonal regulation of lipoprotein lipase activity can be studied. Initial experiments showed that lipoprotein lipase activity is initially low in all cell fractions but undergoes a selective 10-20 fold increase in Fraction I and II cells between the 9th and 12th days of culture. These cells, which are found in the lightest fraction of the gradient (d=1.007-1.070), also

accumulate large intracellular lipid droplets and have an epithelial morphology. They can be propagated in a growth medium which selects against fibroblasts and appear distinct from mammary fibroblasts, which are found in Fraction III of the gradient. The lipase in Fraction I and II cells has been identified as lipoprotein lipase. Thus far, very little lipase activity has been found in the Fraction IV and V cells which appear to be derived from ductal and alveolar secretory elements.

Recent experiments using nutritionally selected Fraction I and II cells have shown that lipoprotein lipase activity is inducible with prolactin plus a limited combination of other hormones. Present studies are directed at determining the specific sequence of hormones events required for the response and confirming the physiological identity of the cells involved. Preliminary findings suggest that there may be a specific cell type in mammary gland which produces lipoprotein lipase in response to hormonal signals prior to parturition and thorughout lactation.

## Pharyngeal Lipase and Digestion of Dietary Fat

Efforts have continued to purify and characterize the lipase present in esophageal fistula juice of man and in the serous glands at the base of the tongue (beneath the circumvallate papilla) in rats. Assay of lipase activity has been facilitated by development of a triglyceride-amphiphilic lipid dispersion in glycerol which after dilution with buffered albumin solution equals natural chylomicrons as a lipase substrate. The stock triglyceride in glycerol preparation currently in use is almost 1 year old and still gives good results.

Mucous in the esophageal juice frustrated early attempts to purify the enzyme. Lyophilization of the juice and denaturation of much of the protein by extraction with acetone and ether made it possible to extract lipase activity with 0.1 M citrate-phosphate buffer, pH 5.4. Gel filtration of the extract on Sephadex G-200 gave 2 peaks of lipase activity - estimated sizes $>2x10^5$ and about $10^5$. Concentration of the collected fractions by ultrafiltration resulted in large losses of activity. Isoelectric focusing of peak II (90k) concentrated the lipase activity at pH 6.5.

Because of the relatively low activity in the esophageal juice preparations we decided to work with the rat tongue enzyme. Passage of active extracts through Sepharose 6B resulted in 2 peaks of activity; one with the void volume, estimated MW of $10^6$ and a second peak of higher specific activity at $2x10^5$. Storage of diluted enzyme solutions at pH 6 results in loss of activity. Freezing and thawing results in loss of activity, >50%. Diluted solutions also tend to be unstable but concentrated solutions, especially the original crude extracts, are stable at 4° for several weeks.

Recent experience with extraction and storage of the enzyme should accelerate progress towards its purification. When sufficient purified enzyme is available, antibodies will be prepared and the identification of the secreting cells established by use of the fluorescent antibody technique. The physiological control of enzyme secretion will then be determined.

## Transport of Lipid, Hormones and Enzymes in Tissues, Cells and Membranes
## Intracellular Membranes and Lipid Formation, Transport and Secretion of Milk Lipid

Electron microscope studies in our laboratory have shown that lipid droplets are intimately associated with intracellular membranes in most cells. These cellular lipid droplets have complex structures composed of homogeneously electron

opaque (osmiophilic material) situated within an irregularly arranged lamellar matrix. In vitro studies on chylomicrons determined that the structure visualized with our preparatory procedures reflect the class of lipid molecules within the chylomicron sphere. Amphiphilic molecules (fatty acids and partial glycerides) formed lamellae at the interface between triacylglycerol and water. These amphiphilic lipids are visualized with the electron microscope as linear densities surrounding homogeneously electron-opaque triacylglycerol.

The structure of lipid droplets seen in thin sections of mammary gland secretory cells similarly reflects the physical properties of the component lipid molecules. The homogeneously electron-opaque areas are triacylglycerol and the lamellar matrix is composed of amphiphilic lipids at the interface between triacylglycerol and the aqueous environment. Further, at the periphery of the lipid droplets, lamellae appear continuous with membrane leaflets of smooth endoplasmic reticulum and Golgi. Thus most of the lamellae within the lipid droplet are thought to be inward extensions of leaflets of membrane bilayers containing triacylglycerol within their hydrophobic hydrocarbon interior. In the lactating mammary gland, the triacylglycerol within leaflets of intracellular membranes is released subsequently to the alveolar lumen as milk lipid. Our structural studies show that in the apical region of the secretory cell, Golgi membrane leaflets enveloping triacylglycerol fuse with the apical plasma membranes releasing the milk lipid package (triacylglycerol and amphiphilic lipid components of the lamellae) to the alveolar lumen.

The significance of these structural findings are: (1) the presence of an interfacial continuum within lipid droplets provides a hydrophobic environment through which presumably liquid triacylglycerol molecules are free to move. Additionally, intracellular translocation of triacylglycerol could be accomplished by an unraveling of lamellae from lipid droplets and their redistribution to other sites within the cell. (2) The interfacial continuum provides also a path for the movement of amphiphilic lipid molecules throughout the cell to sites of esterification. (3) Secretion of milk lipid via the Golgi leaves the apical surface of the alveolar cell covered by Golgi membranes containing specific Golgi enzymes. Such a mechanism dictates that there is, for a finite time, a heterogeneity of enzyme molecules on the cell surface and suggests that molecular redistribution must subsequently occur. (4) The "membrane" fraction of milk contains lamellae which arise from multiple membrane sites within the cell, are heterogeneous in lipid composition and are secreted as a package with the milk triacylglycerol.

Another methodological approach to visualization of lipids is freeze-fracture. Frozen tissue fractures along hydrophobic planes. Therefore structure at sites where membrane leaflets enclose triacylglycerol could be investigated, as well as structure of the cell surface at the sites of membrane fusion. With this technique we avoided chemical fixation of lipids by quick freezing mammary gland. Our initial results show previously unresolved structures within intracellular lipid droplets. There are aggregations of particles on lipid fracture faces suggestive of intracellular particles within membrane leaflets. These particles may represent specific enzyme molecules within monolayers of the lipid droplet. We are investigating also the sequence of reorganization of membrane components during membrane fusion at sites of milk lipid secretion.

## Differentiation of Adipose Cells

Differentiating white adipose tissue from epididymal fat pads of 4 to 6 day old rats was examined with the electron microscope. The presumptive fat pad

contained cells of varying morphology, from spindle-shaped fibroblasts with little lipid to mature unilocular cells. In these cells there is abundant endoplasmic reticulum. Lipid droplets of all sizes contain lamellae and amorphous triacylglycerol. Associations between endoplasmic reticulum leaflets and lipid droplets suggest that lamellae of the lipid droplets are continuous with membrane leaflets. Thus intracellular membranes are the site of both esterification of fatty acid and storage of triacylglycerol within developing adipocytes.

Lipolytic Activity in Liver

In hepatic cells of fed rats, fixed briefly and incubated at 37°C, lipid inclusions showed electron-lucent areas which suggest that enzymatic hydrolysis of triacylglycerol occurs within the droplets. The lamellae present within the droplet may provide the interface for this enzymatic activity.

Transport of Lipid ($^{3}$H-oleate and $^{3}$H-glyceride triacylglycerol) from Capillaries to Parenchymal cells: Electron Microscopic Autoradiography

Previous studies showed that chylomicrons within the capillary bed of lactating rat mammary gland are enveloped by finger-like projections of the capillary endothelium during uptake of triacylglycerol by the tissue. Histochemical studies were interpreted to show that lipolysis of chylomicron-triacylglycerol by lipoprotein lipase occurs at the lumenal surface of the endothelial cell with the release of amphiphilic products (fatty acids and partial glycerides). Electron microscopic autoradiography was used to localize labeled lipid in the tissue, in an attempt to determine the route of transport of chylimicron-fatty acid from the blood to secretory alveolar cells.

Chylomicrons labeled with either glycerol tri[$^{3}$H]oleate glycerol or $^{3}$H-glycerol trioleate were injected into rats lactating 4 days. After 3 minutes mammary tissue was excised and fixed in both osmium tetroxide and/or aldehyde. Some of the aldehyde fixed tissue was further incubated at 37°C in buffer.

Reports in the literature indicate that lipid movement can occur in cells and membranes during and after aldehyde "fixation." This study attempted also to determine if a temporal translocation of lipid in cells of the lactating rat mammary gland occurs after fixation with aldehyde.

The results showed that 3 minutes after injection of chylomicrons into the lactating rat, most of the labeled lipid visualized in the mammary gland, was associated with chylomicrons in the capillary lumen, and also with intracellular and intralumenal milk lipid droplets. Some intracellular movement of label (and thus lipid) occurred during aldehyde fixation.

A Study of Lipid Transport by Lateral Diffusion Using Monolayer Techniques

Our laboratory has been studying for several years the role of lipoprotein lipase and capillary endothelium in the clearance of chylomicrons from blood. On the basis of these studies we recently proposed a model for transport of lipid from blood to tissues by lateral diffusion in cell membranes. According to this model, transport of triacylglycerol from chylomicrons requires conversion of the triacylglycerol by lipoprotein lipase at the capillary surface to acyl lipids - partial glycerides and fatty acids - which locate and move by lateral diffusion in a continuous water-lipid interfacial plane consisting of the chylomicron surface film and the external leaflet of plasma and intracellular membranes of cells. We also proposed that lipolysis of triacylglycerol and movement of lipolytic product from chylomicrons would be enhanced by removal of

the products from the interfacial continuum at some point distal to the chylomicrons, and that such removal would occur primarily in the endoplasmic reticulum of cells as the result of esterification of the lipolytic products to triacylglycerol and accumulation of the latter between the leaflets of the endoplasmic reticulum. Although the experimental techniques used in our studies gave evidence for an interfacial continuum between chylomicrons and endoplasmic reticulum of cells, they were not adequate for studying transport of lipids by lateral diffusion in membranes.

We have recently used lipid monolayer techniques, with the collaboration of Robert Verger of CNRS in Marseille, to study the action of lipoprotein lipase on triacylglycerol, and the movement of lipolytic products by lateral diffusion in a lipid-water interface. In these studies the equivalent of 20 layers of tri-[$^{14}$C]oleoylglycerol was spread over 0.1 M Tris HCl solution, pH 7.4, at 28-30°C under argon. The surface pressure of the interface increased from 15.8 to 18.0 dyne/cm when a small amount (160 µg) of albumin was added to the subphase, and increased further to 20.0 dyne/cm when 10 µg of C-peptides from HDL were added. Addition of 4.4 µg of purified lipoprotein lipase to the subphase caused an immediate and sustained increase in surface pressure to 29 dyne/cm. Since this value is 5-6 dyne/cm less than the collapse pressure for a monolayer of either dioleoylglycerol, monoleoylglycerol or oleic acid, the increase in pressure indicates that trioleoylglycerol was hydrolyzed by lipoprotein lipase and that products of lipolysis displaced trioleoylglycerol and, probably, the protein components from the interface. However, when a large amount (64 mg) of albumin was added to the subphase 2 min after adding lipoprotein lipase, the surface pressure increased only to 24 and then fell to 23 dynes/cm, suggesting that lipolytic products (probably oleic acid and monoleoylglycerol) were being removed from the interface by the large amount of albumin. (Preliminary studies showed that albumin in the subphase could remove oleic acid and, to a lesser extent, monoleoylglycerol but not diacylglycerol from the interface between argon and Tris HCl solution, pH 7.4).

Transport of lipolytic products by lateral movement in the triacylglycerol-water interface was then studied with a trough having two compartments connected by a narrow surface canal. The volume and exposed surface of compartment A were each 3 times that of compartment B. The equivalent of 20 layers of tri-[$^{14}$C]oleoylglylcerol was spread over both compartments; 160 µg of albumin, 10 µg of C-peptide and 4.4 µg of lipoprotein lipase were added to compartment A; and 64 mg of albumin, when used, was added only to compartment B. Aliquots were taken at various intervals from the subphase of each compartment, and analyzed for lipid composition. Hydrolysis of trioleoylglycerol by lipoprotein lipase in compartment A was increased several fold, and movement of oleic acid and monoleoylglycerol to compartment B were increased 5-10 fold when albumin had been added to compartment B. The proportion of lipolytic products recovered in compartment B was also increased, from 16 to 33% of the total, when albumin had been added. When an excess of enzyme was added to A and albumin to B, lipolysis was increased slightly but less than 5% of the products were recovered in compartment B. The latter indicates that very little if any enzyme was present in compartment B, and that the interface above B probably consisted of mostly dioleoylglycerol, which would not be removed by albumin. (Analysis of the inteface above each compartment are being done.)

These studies show that (1) products of hydrolysis of triacylglycerol can locate and move laterally in a lipid-water interface, and that (2) lipolysis and (3) movement in the interface are both enhanced when products of lipolysis are removed from the interface at some point distal to the enzyme. These findings support the model proposed above for the transport of lipid by lateral diffusion in cell membranes.

## PILOT PLANT UNIT

### Large Scale Processing of Biological Materials

The pilot plant under the direction of David Rogerson has processed a total of 393 requests for the culture of various types of organisms. Some of the organisms were used for the production of bacteriophages $T_4$, $T_7$, fd, lambda and PM2, others were used to produce extracellular enzymes, polysaccharides, spheroblasts, and of products of biological importance to numerous laboratories on the NIH campus.

Among the most notable achievements of the pilot plant during this past year was the large scale culturing and processing of lymphocytes for the production of interferon. This was our first attempt to culture animal cells on a massive scale (300 liters thus far but 800 liter cultures are in process). Thus the capacity of the pilot plant to adapt itself to the new developments occuring at NIH has been demonstrated.

With the newly acquired Electro-nucleonics zonal centrifuge, the pilot plant has also updated its ability to process the isolation of enzymes and cell organelles on a large scale and in minimal time. For example, microsomes from twelve lots of chicken livers (10-45 lbs) have been obtained routinely for the purposes of preparing membrane-bound enzymes; viruses have also been isolated in good yield with this instrument. It is anticipated that the zonal centrifuge will be in greater demand now that its usefulness has been demonstrated.

## GERM-FREE ANIMAL UNIT

1. It has been shown that zinc stored in the bones of rats can become available to maintain zinc nutrition during extended periods of zinc-free feeding. The period of normal growth, freedom from deficiency symptoms, and survival time were related to the level of bone zinc, which in turn was related to the amount of zinc administered during a "zinc-loading" period in the first ten days post-weaning.

Bone zinc decreased from relatively high levels throughout the period of observation and reached deficient levels when symptoms became evident.

2. Germfree-rats appear to require less zinc or to conserve it more efficiently than rats with bacteria. Female rats appear to require less zinc than males. This sex difference is more marked in germfree than in non-germfree animals.

We have been able by dietary means to produce a rat exhibiting severe symptoms of tryptophan deficiency, which include tumors and a marked hypersensitivity to stimulation by mechanical stimuli. Levels of 5-hydroxytryptamine in brain and other organs were found to be depressed in these deficient animals. This animal can be produced within a period of thirty days and hopefully will be a suitable model to further study the abnormal behavior.

| SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space) | U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT | PROJECT NUMBER Z01 AM 15000-07 LNE |
|---|---|---|

PERIOD COVERED
July 1, 1976 through September 31, 1977

TITLE OF PROJECT (80 characters or less)
Mechanism of Action of Hormones and Guanine Nucleotides on Adenylate Cyclase Systems

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: Martin Rodbell Chief, LNE, NIAMDD

COOPERATING UNITS (if any)
M. Berman and M. Rendell (NCI)

LAB/BRANCH
Laboratory of Nutrition and Endocrinology, NIAMDD

SECTION
Membrane Regulation

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20014

| TOTAL MANYEARS: | PROFESSIONAL: | OTHER: |
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| 0.5 | 0.5 | |

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

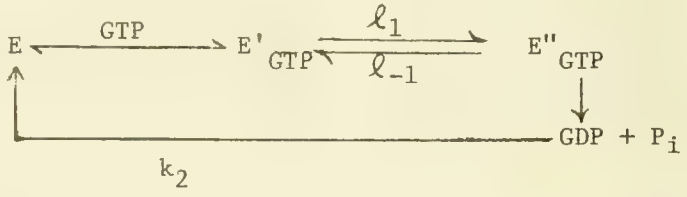
The transient kinetics of guanine nucleotide activation of adenylate cyclase has been modeled with the SAAM modelling program. The model developed extends the previously reported three state model and provides an explanation for the differences both in effectiveness and kinetics observed with GTP and Gpp(NH)p. In essence, the model suggests that, in addition to the catalytic site that converts ATP to cyclic AMP, the enzyme system contains a GTPase at the nucleotide regulatory site that converts GTP to inactive product, GDP. One important outgrowth of the model is that is serves to explain the rapidly reversible dynamic features of hormone and GTP activation of adenylate cyclase. As related in a separate report, it also serves to explain the actions of cholera toxin on adenylate cyclase systems.



PHS-6040

Project Description

Modeling of the transient kinetics of Gpp(NH)p- and GTP-activation of the hepatic adenylate cyclase system was carried out with the SAAM program developed by Dr. Mones Berman. Using the kinetic parameters obtained at steady state from modeling of the Gpp(NH)p action and the appropriate rate equations for the three state model, it was determined that the transient kinetics of Gpp(NH)p-activation could be reproduced, thus validating the considerations of the three state model published previously. The three state model was then extended to explain why GTP is much less active than Gpp(NH)p and, unlike the latter nucleotide, does not show transient (i.e., "lags" in onset of activation) kinetics. Various models were considered to explain these differences; the data could be stimulated by a model which suggests that, in addition to the catalytic site that converts ATP to cyclic AMP, the enzyme system (E) contains a catalytic site which degrades GTP to the inactive product, GDP, as follows




In this model, the activity of the GTPase is not expressed until the system is converted to E" (the high activity state of adenylate cyclase): $k_2$ is the rate constant for the breakdown of GTP to GDP and the reversion of E" back to the ground state, E; $\ell_1$ and $\ell_{-1}$ are the forward and reverse rate constants for the transition between states E' and E". The model makes three fundamental predictio One is that any agent which increases $\ell_1$ should increase the concentration of E"·GTP at steady state, thus resulting in both an increase in the activity of adenylate cyclase as well as an increase in the breakdown of GTP through the GTPase site. Our previous studies with the fat cell and liver adenylate cyclase systems showed that hormones (glucagon, ACTH, epinephrine, secretin) increase $\ell_1$. Studies reported recently with the turkey erythrocyte system showed that catecholamines, through the beta receptor, increase the breakdown of GTP to GDP+$P_i$ concomitantly with an increase in cyclic AMP production from ATP. Thus, the predictions of the model have been borne out with experimental evidence.

The second prediction is that agents which block GTPase activity associated with state E", should allow GTP to become as potent an activator as GTP analogs which are not hydrolyzed at the terminal phosphate (Gpp(NH)p, Gp($CH_2$)p). Evidence that cholera toxin $A_1$ is such an agent has recently been obtained in our laboratory by Dr. Lin.

A third prediction is that agents which accelerate the breakdown of GTP at the GTPase site should inhibit adenylate cyclase activity. No experimental evidence with any agent of this category has been obtained as yet. It is of interest, however, that adenosine inhibits the hepatic adenylate cyclase at a site that is independent of the various regulatory sites examined thus far. The inhibitory effect is particularly evident in the presence of glucagon, GTP, and metal ions, but not in the presence of Gpp(NH)p. The possibility can be considered, therefore, that adenosine is an agent that promotes GTP breakdown at the GTPase site.

Until the discovery of the nucleotide regulatory site, regulation of adenylate cyclase activity was confined primarily to the role of hormones in this process. The discovery of the actions of guanine nucleotides on the enzyme system revealed new sites of regulation and indeed has provided a functional and possibly structural linkage between the receptor and the enzyme. The concept of a GTPase that controls the turnover of GTP at the nucleotide regulatory site introduces another level of control of this complex regulatory enzyme system in which hormones, in a seeming paradoxical sense, stimulate both the production of cyclic AMP as well the destruction of the essential activator, GTP. Thus hormone action through its receptor and the turnover of GTP through the nucleotide regulatory site and GTPase can be described as a system that oxcillates between low and high activity states of activity determined by the rate of turnover of GTP and the accessibility of GTP, presumably supplied by the cell, to the system.

The modeling of the transient kinetics of guanine nucleotide activation of adenylate cyclase has contributed, therefore, to our understanding of the regulation of adenylate cyclase, one of the most important regulatory enzymes known in biological systems. The concept of a GTPase raised by the model must be tested by direct demonstration of the GTPase activity. Such studies are in progress.

Publications:

None

| SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space) | U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT** | PROJECT NUMBER ZOL AM 15001-03 |
|---|---|---|

PERIOD COVERED

July 1, 1976 through September 31, 1977

TITLE OF PROJECT (80 characters or less)

Structure-Function Relationship of Glucagon

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

| | | | |
|---|---|---|---|
| PI: | Michael C. Lin | Research Chemist | LNE NIAMDD |
| OTHER: | David E. Wright | NIH Fellow | LNE NIAMDD |

COOPERATING UNITS (if any)

Dr. V. J. Hruby University of Arizona

LAB/BRANCH

Laboratory of Nutrition and Endocrinology

SECTION

Section on Membrane Regulation

INSTITUTE AND LOCATION

NIAMDD, NIH, Bethesda, Maryland 20014

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The goal of this project is to determine the structural requirements of glucagon for binding to its receptors and for its action on the adenylate cyclase system. In order to avoid side reactions occurring at the ε-amino group, the lysyl residue in glucagon was converted to homoarginine. The resultant derivative, (homoArg$^{12}$)-glucagon, provided an ideal precursor for subsequent modifications at the amino-end of glucagon. (Des-His$^{1}$, homoArg$^{12}$)glucagon was prepared and purified. Initial findings confirmed that removal of the amino-terminal histidyl residue produced a partial agonist. Preliminary studies indicate that replacing histidine with N-acetyl homocysteine in the glucagon regenerated substantial activity, which was previously lost die to removal of the histidyl residue. This finding suggests that while the amino terminal residue of glucagon is important to its activity, histidine itself does not play an essential role. Other derivatives of interest will be prepared and studied to further elucidate the structural requirements for glucagon.



PHS-6040
(Rev. 10-76)

Project Description

Objective: The goal of this project is to determine the structural requirements for glucagon which are necessary in receptor binding and the subsequent biological response. New understanding in these events can be gained by studying the effects of chemical modification on the native hormone.

Methods: (HomoArg$^{12}$)-glucagon was prepared by reacting the lysyl residue of native glucagon with O-methylisourea, and the subsequent removal of the histidyl residue with phenyl isothiocyanate produced (des-His$^{1}$, homoArg$^{12}$)-glucagon. (N-acetyl homoCys$^{1}$, homoArg$^{12}$)-glucagon was prepared by the reaction of N-acetyl homocysteine thiolactone with (des-His$^{1}$, homoArg$^{12}$)-glucagon. The biological activity of these glucagon derivatives was determined by their ability to activate hepatic adenylate cyclase, and binding was measured by the interaction with receptors in the hepatic plasma membranes.

Major Findings: Previous studies suggest that the amino terminal region of glucagon plays an essential role in its activity, however, modifications at the α-amino group have been hampered due to side reactions occurring at the ε-amino group of lysyl residue. This problem has now been overcome by converting the lysyl residue to homoarginine which unreactive toward most of the reagents specific for amino groups. (HomoArg$^{12}$)-glucagon is almost fully active and can be prepared in large quantities, therefore, it provides an ideal precursor for subsequent modification at the amino -end of glucagon. The aminoterminal histidine residue of glucagon was removed by phenyl isothiocyanate, and the resultant derivative, (des-His$^{1}$, homoArg$^{12}$)-glucagon, is a partial agonist just like the previously prepared (des-His$^{1}$)-glucagon. A preliminary study showed that the incorporation of N-acetyl homocysteine in place of His$^{1}$ regenerated substantial activity previously lost due to removal of the His$^{1}$ residue. This finding suggests that while the amino terminal residue of glucagon is important to its activity, histidine itself does not play an essential role.

Significance to Biomedical Research and the Program of the Institute: Knowledge of the structural requirements of glucagon with regards to function is fundamental to the understanding of the hormone's action. This is especially important since recent findings implicate glucagon playing a major role in the disease diabetes.

Proposed Course of Project: Preparation of (homoArg$^{12}$)-glucagon provides a new approach to the modification of the amino terminal region of glucagon. However, the purity of this derivative is now only 95%; it is essential that 99% purity be obtained in order avoid ambiguity in the interpretation of subsequently prepared derivatives. Scaled-up preparation and further purification of (homoArg$^{12}$)- and (des-His$^{1}$, homoArg$^{12}$)-glucagons are the major objectives at the present time. Whether (N-acetyl homoCys$^{1}$, homoArg$^{12}$)-glucagon is a full agonist and how it interacts with the receptors remain to be studied. Other derivatives of interest will be prepared and studied to further elucidate the structural requirements for glucagon.

Publications:

Lin, Michael C., Nicosia, Simonetta and Rodbell, Martin: Effect of Iodination of Tyrosyl Residues on the Binding and Action of Glucagon at its Receptor. Biochemistry 15: 4537-4540, 1976.

Hruby, V.J., Wright, D.E., Lin, M.C. and Rodbell, M.: Semisynthetic Glucagon Derivatives for Structure-Function Studies. Metabolism 25: 1323-1325, 1976.

| SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space) | U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT** | PROJECT NUMBER ZO1 AM 15002-03 |
|---|---|---|

PERIOD COVERED

July 1, 1976 through September 31, 1977

TITLE OF PROJECT (80 characters or less)

Studies on Binding of $^{3}$H-glucagon to Receptors in Rat Hepatic Plasma Membranes

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: Michael C. Lin Research Chemist LNE NIAMDD

COOPERATING UNITS (if any)

NONE

LAB/BRANCH

Laboratory of Nutrition and Endocrinology

SECTION

Section on Membrane Regulation

INSTITUTE AND LOCATION

NIAMDD, NIH, Bethesda, Maryland 20014

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

In order to avoid the problems introduced by iodine incorporation into glucagon, $^{3}$H-glucagon was prepared and was used to reexamine the kinetic relationship between binding and action of glucagon. The results showed that the time courses of glucagon binding to its receptors and of activation of adenylate cyclase correlate closely. This study also revealed that, in the presence of GTP, a high affinity state of the receptors exists, in addition to the low affinity state detected using $^{125}$I-glucagon in the previous study. These findings provided new insight on the overall organization and regulation of the adenylate cyclase system. This project has been completed and the findings were published.



PHS-6040
(Rev. 10-76)

Project Description

Objectives: In view of the problem associated with the use of $^{125}$I-glucagon as reported previously, highly purified $^{3}$H-glucagon was prepared. The goal of this project is to reexamine the hormone binding to its receptors and to reevaluate the kinetic relationship between binding and action of glucagon.

Methods: Iodoglucagon was prepared by chloramine T procedure followed by purification with column chromatography. $^{3}$H-glucagon was prepared by catalytic reduction of iodoglucagon with tritium gas and followed by extensive purification. The biological activity was determined by the adenylate cyclase assay. Binding was measured by the interaction of glucagon with its receptors in the hepatic plasma membranes.

Major Findings: Extensive characterization of $^{3}$H-glucagon showed that it behaved similarly to native hormone in its binding and activity. By using this radioactive glucagon we showed that the time course of binding to its receptors correlates closely, in the presence of GTP, with the time course of activation of adenylate cyclase by native hormone. The study also revealed that in the presence of nucleotide, a high affinity state of the receptor exists in addition to the low affinity state detected using $^{125}$I-glucagon in the previous study. These findings provided new insight on the relationship between the hormone receptors and nucleotide components and their involvement in the overall organization of the adenylate cyclase system.

Significance to Biomedical Research and the Program of the Institute: Characterization of hormone receptors in the responding system is fundamental to the understanding of hormone response. The use of $^{3}$H-glucagon clarified a few perplexing disparities previously reported between the binding and action of glucagon. These new findings allowed us to reformulate our thinking toward the overall regulation of cyclic AMP-mediated hormones.

Proposed Course of Project: This project has been completed and the findings were published as listed below.

Publications:

Lin, Michael C., Nicosia, Simonetta, Lad, Pramod M. and Rodbell, Martin: Effects of GTP on Binding of $^{3}$H-glucagon to receptors in Rat Hepatic Plasma Membranes. J. Biol. Chem. 252: 2790-2792, 1977.

| SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space) | U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT | PROJECT NUMBER ZO1 AM 15004-02 |
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PERIOD COVERED
July 1, 1976 through September 31, 1977

TITLE OF PROJECT (80 characters or less)

Studies on Hormone Responses in Cultured Cells

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

| | | | |
|---|---|---|---|
| PI: | Michael C. Lin | Research Chemist | LNE NIAMDD |
| OTHER: | Chiu-shiong Lin | Visiting Fellow | LNE NIAMDD |

COOPERATING UNITS (if any)

NONE

LAB/BRANCH
Laboratory of Nutrition and Endocrinology

SECTION
Section on Membrane Regulation

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland, 20014

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The ultimate goal of this project is to study the regulation of hormone response by extracellular enviroment and by the target cells themselved. A long term cell culture which retains its hormone sensitivity has been developed from fetal rat liver. Studies on cyclic AMP mediated hormones such as glucagon and Epinephrine have been emphasized initially, because of their relatively well established mode of action. One culture condition has been observed, which causes acute disappearance of hormone responses. This observation is being studied to determine whether the loss of hormone sensitivity is related to culture conditions and whether it is relevant to regulation of cell metabolism.



PHS-6040
(Rev. 10-76)

Objectives: The goal of this project has been broadened to include studies of cyclic AMP-mediated hormones, in addition to studies of growth hormone. Through the use of a hormonally-sensitive cell culture, factors controlling responsiveness of hepatocytes are being studied. The major objective of this project is to understand how cells and culture environment regulate the hormone response and to further understand the responding system itself.

Method Employed: A cell culture has been developed from fetal rat liver and maintained in a rich medium. Hormone response is measured in terms of cyclic AMP production in the cases of glucagon and epinephrine, and leucine incorporation for growth hormone. Binding of radioactive hormone is used to quantitate the presence of receptor. Other types of activities, characteristic of hepatocytes, such as albumin synthesis and level of glucose-6-phosphatase, are also measured to monitor the fidelity of the cell line.

Major Findings: Responsiveness to glucagon and epinephrine can be preserved for longer than one month in a primary and secondary culture obtained from fetal rat liver. Whether this loss of sensitivity is associated with loss of hormone receptor is currently under investigation. However, this phenomenon does not seem to be related to the so called "desensitization or down regulation" of hormone response. Guanine nucleotide activates adenylate cyclase system alone or synergistically with hormone, its activating effect is enhanced by at least two-fold after hepatocytes were placed in culture for few days. The significance of this observation is under study.

Significance to Biomedical Research and the Program of the Institute: Control of hormone response by cells and their environment is a relatively new aspect of regulation of life process requiring extensive study. A detailed knowledge of this mechanism is essential to the full understanding of biological system. A long term cell culture, which is hormonally sensitive and maintains differentiated characteristics of liver function, is obviously an invaluable tool for research in endocrinology.

Proposed Course of Project: Variables involved in cell culture are numerous. We are still in the process of optimizing the condition for preservation of hormone response in the hepatocyte culture. Enhanced guanine nucleotide activation of adenylate cyclase system and the acute disappearance of hormone sensitivity will be studied in detail to determine whether these observations are related to culture condition and whether they are relevant to regulation of cell metabolism.

| SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space) | U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT** | PROJECT NUMBER ZO1 AM 15005-02 LNE |
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PERIOD COVERED
July 1, 1976 through September 31, 1977

TITLE OF PROJECT (80 characters or less)

Regulation of Adenylate Cyclase by Adenosine

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: C. Londos Senior Staff Fellow LNE NIAMDD

COOPERATING UNITS (if any)

J. Wolff CEB NIAMDD

LAB/BRANCH
Laboratory of Nutrition and Endocrinology

SECTION
Section on Membrane Regulation

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20014

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| 0.4 | 0.3 | 0.1 |

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The actions of adenosine on several adenylate cyclase systems have been investigated. Adenosine inhibits hepatic adenylate cyclase at a site which, based on analog studies, is markedly different from the site through which the nucleoside activates the Leydig cell cyclase. The adenylate cyclase in platelets contains two sites for adenosine action which appear identical to the sites identified in liver and Leydig cells. Thus, adenylate cyclase systems may contain one or the other, or both types of adenosine receptor. These studies provide a basis for testing whether any of the many biologic effects of adenosine are mediated by adenosine acting through the adenylate cyclase system.



PHS-6040
(Rev. 10-76)

Project Description

Objectives: Adenosine at micromolar concentrations has varying effects on adenylate cyclase activity. Depending on the source of the enzyme, the nucleoside may activate or inhibit activity, and in some cases both activation and inhibition are observed with a single adenylate cyclase. It was the goal of these studies to determine, with the use of adenosine analogs, whether these multiple effects are mediated by similar or different adenosine receptor sites.

Methods: Membranes were isolated from various tissues and adenylate cyclase was assayed by standard procedures.

Major Findings: We have strong evidence indicating that the effects of adenosine are mediated by at least two markedly different "receptors".

| Analogs which inhibit liver cyclase but do not activate Leydig cell cyclase. | Analogs which activate Leydig cell cyclase but do not inhibit liver cyclase. |
|---|---|
| 2-methyladenosine | 2',5'-dideoxyadenosine |
| 2-aminoadenosine | 2'-deoxyadenosine |
| $N^6$-methyladenosine | 2,3'-isopropylidene adenine-D-arabinoside |
| $N^6$-phenylisopropyladenosine | adenine-D-xyloside |
| Purine-D-riboside | |
| formycin | |

The above table shows the effects of adenosine analogs on two cyclase systems, that from rat liver, which is ihhibited by adenosine, and that from Leydig tumor cell, which is activated by the nucleoside. It is seen that the adenosine site on the rat liver enzyme tolerates changes in the ribose ring but is highly sensitive to alterations in the purine moiety. The opposite is true for the adenosine site associated with the Leydig cell cyclase. With the latter, changes in the 2'-hydroxyl result in inactive compounds.

We then examined the enzyme from human platelets on which adenosine produces a biphasic effect, that is, activation at low concentrations and inhibition at higher concentrations. Based on analogs studies it appears that these two effects are mediated by two different sites, and the two sites are similar, if not identical, to the two different sites identified in the liver and Leydig cell. That is, the analogs that stimulate the Leydig cell cyclase also stimulate the platelet enzyme, and the analogs that inhibit the liver system also inhibit the platelet enzyme.

The above studies have identified two strikingly different adenylate cyclase-associated adenosine "receptors" and revealed that, depending on the tissue studied, cyclases may have one or the other receptor, and in some cases, both receptors on a single enzyme.

Significance to Bio-Medical Research and the Program of the Institute. Adenosine, at concentrations which may be considered physiological, has effects on biologic systems ranging from increased coronary blood flow to suppression of the immune response in lymphocytes. There are indications, but no firm evidence, that the nucleoside effects result from its ability to alter cellular cyclic AMP levels. Our studies provide a basis for testing whether adenosine effects are mediated by adenylate cyclase.

Proposed Course of Project: Our immediate goal is to find antagonists of adenosine action at both of the sites we have identified. This will be done either by testing the available pool of adenosine analogs or, based on the structural mapping of the two sites, by having synthesized compounds likely to be blockers of adenosine action. With the use of appropriate antagonists we then plan to test whether the effects of adenosine on several biologic systems are mediated by adenylate cyclase.

Publications:

Londos, C. and Rodbell, M.: Adenylate Cyclase: Actions and Interactions of Regulatory ligands. In: Drug Action at the Molecular Level (Ed.G.C.K. Roberts) London, Macmillan Press; 235-247 (1977).

Londos, C. and Preston, M.S.: Activation of the Hepatic Adenylate Cyclase System by Divalent Cations: A Reassessment; J. Biol. Chem. (In Press)

Londos, C. and Preston, M. S.: Regulation by Glucagon and Divalent Cations of Inhibition of Hepatic Adenylate Cyclase by Adenosine; J. Biol. Chem. (In Press).

Rodbell, M. and Londos, C.: Regulation of Hepatic Adenylate Cyclase by Glucagon, GTP, Divalent Cations, and Adenosine; Metabolism 25 (Suppl. 1) 1347-1349 (1976).

| SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space) | U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT** | PROJECT NUMBER Z01 AM 15006-02 LNE |
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PERIOD COVERED
July 1, 1976 through September 31, 1977

TITLE OF PROJECT (80 characters or less)

Purification and Characterization of Hepatic Adenylate Cyclase

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: Rodbell, M.

| | | |
|---|---|---|
| OTHER: | Lad, P.M. | Visiting Fellow |
| | Welton, A.E. | Guest Worker |
| | Newby, A. | Guest Worker |
| | Yamamura, H. | NIH International Fellow |

COOPERATING UNITS (if any)

None

LAB/BRANCH
Laboratory of Nutrition and Endocrinology

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Membrane Regulation

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Adenylate cyclase and the glucagon-receptor complex have been solubilized from rat liver plasma membranes with Lubrol. The solubilized material was chromatographed on ultrogel columns to give about five-fold purification of the enzyme. The glucagon-receptor complex separated from the enzyme on the column. Both enzyme and receptor-hormone complex remained susceptible to the actions of guanine nucleotides, suggesting that the receptor and enzyme were each associated with a guanine nucleotide regulatory site. This possibility was investigated further with studies on the membrane-bound forms of these components. It was found that the effects of guanine nucleotides on receptor and enzyme were exerted through functionally different processes as revealed by differences in potency of analogs of GTP, different effects of chelators and phospholipases on the two processes, and differences in reversibility of the actions of Gpp(NH)p on enzyme activity and receptor binding. These findings have been incorporated into a kinetic model that may explain the role of the nucleotide regulatory components in the "coupling" of receptor and enzyme, and in the overall regulation of enzyme activity.

Project Description

Previous studies showed that hepatic adenylate cyclase, when solubilized with Lubrol, could be purified about 5-fold by chromatography on Ultrogel ACA22 and ACA34 columns. The partially purified, solubilized enzyme retained the characteristics of Gpp(NH)p-activation seen with the membrane-bound form of the enzyme. By following the binding of $^{3}$H-Gpp(NH)p to various column fractions it was determined that less than 1% of the total Gpp(NH)p-binding proteins present in hepatic membranes could be associated with the process of Gpp(NH)p-activation of adenylate cyclase. Based on the total Gpp(NH)p-binding sites in the membranes, a maximum of 0.5 pmoles of Gpp(NH)p-binding sites represent the enzyme. This value contrasts with about 5 pmoles of glucagon receptor molecules in the membrane based on studies of the binding of labeled glucagon to plasma membranes. Thus, there appears to be an excess of receptors over enzyme in the hepatic plasma membrane.

We had also shown in previous studies of the binding of glucagon to hepatic membranes that GTP converts all of the glucagon receptors to at least two binding states: one state has an affinity constant greater than 10 nM which is far higher than the estimated $K_a$ for glucagon-activation of adenylate cyclase and is likely not to represent the coupled receptor-adenylate cyclase state of the enzyme system. This low affinity state formed by GTP encompasses about 80-90% of the total receptor states formed by the nucleotide. A second state formed with GTP shows a high degree of cooperative binding (Hill coefficient greater than 2) with a $K_d$ in the range expected for the coupled form of the receptor; this state comprises from 10-15% of the total receptor states formed with GTP. From these findings, it appears that only a fraction of the receptors (10-15%) become coupled to adenylate cyclase in the presence of glucagon and GTP. However, GTP affects the binding of glucagon to its receptors whether the receptors are coupled or not to the enzyme. Since GTP is also necessary for activation of adenylate cyclase in the presence or absence of hormone receptors, the possibility was considered that both the glucagon receptor and adenylate cyclase were each linked to nucleotide regulatory sites or components that interact with GTP.

To test for this possibility, several approaches were employed. The glucagon-receptor complex formed with intact membranes could be solubilized with Lubrol, separated from adenylate cyclase on Ultrogel columns; it remained susceptible to GTP action even though separated from adenylate cyclase in its nucleotide sensitive form. Other approaches were used with the intact membrane. It was found that the potencies of GTP and various analogs of GTP were markedly different on the binding of glucagon to receptor and activation of adenylate cyclase. Moreover, these processes were effected differentially by helators, by phospholipase, and displayed markedly different characteristics with respect to reversibility of action. Based on the above findings, it was concluded that the glucagon receptor contains a separate nucleotide regulatory site from the regulatory site that controls adenylate cyclase activity, that the receptor is separate from adenylate cyclase and is about 10-fold higher in concentration than the enzyme in hepatic membranes.

A kinetic model was proposed which suggests that the concentration of the high activity, receptor-coupled state of the hepatic adenylate cyclase system is determined not only by the small regulatory ligands, GTP and glucagon, but

also by the concentrations of the macromolecular components, receptor and enzyme involved in the formation of the active state. The findings which suggest that receptor and enzyme each contain a nucleotide regulatory site raise the possibility that the nucleotide regulatory components, which are likely to be facing the cytoplasmic side of the plasma membrane, may be involved in the linkage of the receptor and enzyme in the membrane.

The model raises a number of interesting questions that require further testing. The obvious prime questions are the nature of the nucleotide regulatory components and how they are linked to receptor and enzyme. The projected course of study for the coming year is to establish means of identifying the guanine nucleotide site and of determining whether these components can be separated from the enzyme and receptor. This project is stymied at the moment because of the low concentration of relevant nucleotide regulatory components. We are attempting to develop affinity analogs of GTP (or Gpp(NH)p) that may selectively "tag" the relevant sites. Another approach being pursued it to develop cultured lines of hepatic cells which may lack a hormone-sensitive cyclase system but which contain, nonetheless, hormone receptors and enzyme. If the hormone receptors in such cell lines do not demonstrate a functional linkage to guanine nucleotide sites, we would then have a genetic handle to the problem. Hopefully, a number of different approaches will become apparent as we develop the necessary techniques for identifying the nucleotide sites.

The importance of the findings reported here is that a coherent pattern is emerging from our studies with glucagon and from the studies of others with different hormones that guanine nucleotides are essential for both the activation of adenylate cyclase and the regulation of enzyme activity by hormones. The evidence suggesting two separate nucleotide regulatory components offers, for the first time, the means of explaining the "coupling" of receptors and enzyme within the membrane and may, in addition, provide information on the structural features of hormone-sensitive adenylate cyclase systems, systems that have proven to have a major role in biological systems and therefore an important role in biomedical problems.

Publications:

Yamamura, H., Rodbell, M. and Fain, J.N: Hydroxybenzylpindolol and Hydroxybenzylpropranolol: Partial Beta Adrenergic Agonists of Adenylate Cyclase in the Rat Adipocyte. Mol. Pharmacology 12 693-700, 1976.

Welton, A.E., Lad, P.M., Newby, A.C., Yamamura, H., Nicosia, S., and Rodbell, M: Solubilization and Separation of the Glucagon Receptor and Adenylate Cyclase in Guanine Nucleotide-Sensitive States J. Biol. Chem. (In Press).

Lad, P.M., Welton, A.E., and Rodbell, M.: Evidence for Distinct Guanine Nucleotide Sites in the Regulation of the Glucagon Receptor and of Adenylate Cyclase Activity J. Biol. Chem. (In Press).

Yamamura, H., Lad, P.M., and Rodbell, M.: GTP Stimulates and Inhibits Adenylate Cyclase in Fat Cell Membranes Through Distinct Regulatory Processes J. Biol. Chem. (In Press).

| SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space) | U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT** | PROJECT NUMBER Z01 AM 15007-01 LNE |
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PERIOD COVERED
July 1, 1976 through September 31, 1977

TITLE OF PROJECT (80 characters or less)

Purification of Testicular Adenylate Cyclase

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

| | | | |
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| PI: | C. Londos | Senior Staff Fellow | LNE NIAMDD |
| | A.M. Geller | Visiting Scientist | LNE NIAMDD |
| | T. Shinozawa | Guest Researcher | LNE NIAMDD |

COOPERATING UNITS (if any)

None

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Laboratory of Nutrition and Endocrinology

SECTION
Section on Membrane Regulation

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20014

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

We are attempting to purify a soluble form of adenylate cyclase present in the mature rat testis; though soluble, it is conceivable the primary structure of the soluble testis enzyme is similar to or even identical to the adenylate cyclase bound to membranes. Antibodies to purified test enzyme may interact, for example, with the hepatic adenylate cyclase and thus allow, with appropriately established methods quantitation of the amount of hepatic enzyme, perhaps localize the enzyme in its membrane form, and purify, on antibody addinity columns, the enzyme after solubilization. We have succeeded in purifying the testis enzyme 500-fold with the use of $(NH)_4)_2SO_4$ precipitation, DEAE-argarose chromatography, Sephadex G-150 chromatography, ATP-agarose affinity columns. Hydroxy-apatite columns have recently given another 10-fold purification. Thus we are now in position to develop antibodies to the testis enzyme for the purposes outlined above.



PHS-6040
(Rev. 10-76)

Project Description

Objectives: Adenylate cyclase systems in eukaryotic cells play an important if not essential role in the expression of hormone action. The cyclases which respond to hormones are embedded in plasma cell membranes and have, thus far, proved intractable to purification. At this time there is no method for detection of adenylate cyclase other than measuring for enzymatic activity, and the enzyme is labile to forces required to separate it from other membrane proteins. Also, there is at present no way to quantitate the adenylate cyclase content in cells.

Mature rat testes produce a soluble adenylate cyclase which should be amenable to standard protein purification procedures. It is the goal of this project to purify and produce antibodies to the testicular enzyme. If such antibodies cross-react with the membrane-bound form of the enzyme it would permit detection and quantitation of cellular adenylate cyclase and, with the use of affinity chromatography, provide a means for purifying the membrane-bound enzyme.

Major Findings: With respect to testicular homogenate activity the adenylate cyclase has been purified approximately 500-fold to a specific activity of 0.4 micromol cyclic AMP formed per mg prot per min with the use of $(NH_4)_2SO_4$ precipitation, chromatography on DEAE-agarose, Sephadex G-150, and ATP-agarose. We have recently overcome certain problems resulting from exposure of the enzyme to phosphate and now find that chromatography on hydroxylapatite give an additional 10-fold purification. We are currently processing, on a large scale, the testicular cyclase with the above-mentioned procedures plus hydroxylapatite, which should yield the pure protein.

Significance to Bio-Medical Research and the Program of the Institute: The molecular dissection of hormonally-responsive membrane-bound adenylate cyclase systems would provide information on the manner by which hormones transmit signals across plasma cell membranes. Conventional procedures for purifying soluble enzymes have thus far been ineffective in purifying membrane-bound adenylate cyclase. Should the antibody to the soluble testicular enzyme react with the membrane-bound form, this project will have provided a new and potentially powerful tool for the purification effort. Other possible benefits would include a means for quantitating the enzyme and assessing the relationship between hormone receptors and the cyclase.

Proposed Course of the Project: We plan to continue to purify the testicular enzyme and to produce antibodies to the enzyme.

Publications: None.

| SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space) | U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT** | PROJECT NUMBER Z01 AM 15008-01 LNE |
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PERIOD COVERED

July 1, 1976 through September 31, 1977

TITLE OF PROJECT (80 characters or less)

Reversible Activation of Adenylate Cyclase by GTP analogs

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

| | | | |
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| PI: | C. Londos | Senior Staff Fellow | LNE NIAMDD |
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| | A.F. Welton | NIH Fellow | LNE NIAMDD |
| | P.M. Lad | NIH Fellow | LNE NIAMDD |
| | M. Rodbell | Chief | LNE NIAMDD |

COOPERATING UNITS (if any)

None

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NIAMDD, NIH, Bethesda, Maryland 20014

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Activation by hormone of adenylate cyclase is dependent on the presence of guanine nucleotides which act at a nucleotide regulatory site. The actions of GTP at the regulatory site are readily reversible, in contrast to the actions of analogs such as Gpp(NH)p and Gpp($CH_2$)p which produce persistent activation. We have found that the α-β methylene analog, Gp($CH_2$)pp, is also an effective activator of hepatic adenylate cyclase and exhibits characteristics similar to Gpp(NH)p. The stability of the α-β methylene bond makes it highly unlikely that the so called "persistent" activation results from covalent modification of the enzyme by guanine nucleotides, as has been suggested in the literatures. We have also shown that activation by these analogs is slowly reversible, which suggests that the persistence of activation results from tight binding of the analogs to the nucleotide regulatory site. The fact that all of the analogs which produce the slowly reversible activated state are stable to hydrolysis at the β-γ position suggests that the regulatory site contains a GTPase.

Project Description

Objectives: The actions of numerous hormones on target cells is mediated by adenylate cyclase. Activation of the enzymes by hormone is highly dependent upon the presence of guanine nucleotides which act at a regulatory site to, depending on the system studied, permit or enhance hormone action. The actions of GTP at the regulatory site are rapidly reversible, which is in contrast to the effects of certain GTP analogs with C or N substitutions between the beta and gamma phosphates($Gpp(CH_2)p$ and Gpp(NH)p). These analogs activate adenylate cyclases in an apparently "irreversible" manner. Two explanations have been advanced to explain this persistent activation: 1) the enzyme is "pyrophosphorylated" to form $E\text{-}p(CH_2)p$ or E-p(NH)p which are resistent to phosphohydroase activity, in contrast to E-pp formed with GTO and 2) the analogs bind tightly to and dissociate slowly from the regulatory site. The goal of these studies was to determine, with the use of a new analog, $Gp(CH_2)pp$, whether the "pyrophosphorylation" hypothesis is tenable and, if not, whether reversibility of the persistent activation could be demonstrated.

Methods: Membranes were isolated and adenylate cyclase assayed by standard procedures established in this laboratory.

Major Findings: $Gp(CH_2)pp$ is highly resistent to liver membrane phosphohydrolase and pyrophosphohydrolase activity under conditions which result in nearly complete hydrolysis of GTP and considerable hydrolysis of Gpp(NH)p. The new analog is highly stable not only at the $\alpha$-$\beta$ methylene but at the $\beta$-$\gamma$ P-O-P. Despite its stability, $Gp(CH_2)pp$ activates hepatic adenylate cyclase in a manner highly reminiscent of Gpp(NH)p. That is, there is a lag in onset of activation, and after treatment with $Gp(CH_2)pp$ and washing of membranes to remove the compound the enzyme remains in an activated state. Given the stability of $Gp(CH_2)pp$ and the great unlikelihood that the analog would serve as a substrate for "pyrophosphorylation", we conclude that activation does not involve covalent modification of adenylate cyclase.

The likely alternative explanation for the persistene activation is that the nucleotides bind tightly to and dissociates slowly from the enzyme, and we have been able to demonstrate that activation by the analogs, Gpp(NH)p and $Gp(CH_2)pp$, is in fact reversible. In order to demonstrate reversibility we took advantage of the fact that GTP is a much weaker activator than either of the two analogs. Following the achievement of steady state in the presence of the analogs, the addition of GTP results in a slow decay to the rate given by GTP alone. This decay is barely noticeable after 3 min, and at least 6 min are required before steady state is achieved. We have also shown that the enzyme, once activated and permitted to decay, can again be re-stimulated with the analogs.

The GDP analog, $Gp(CH_2)p$, binds to the nucleotide regulatory site but does not activate the enzyme. This could be demonstrated only when phosphate donors (ATP and nucleotide-regenerating systems) were omitted from the assay medium. Thus, the gamma phosphate of the guanine nucleotide is required for activation.

Major Findings (Continued):

From the above studies we conclude that guanine nucleotides activate adenylate cyclases by binding to an allosteric site. The slow reversibility of activation seen with analogs stable to hydrolysis at the beta-gamma bond, in contrast to GTP, suggest that the regulatory site normally terminates the GTP message by hydrolyzing the nucleotide to inactive GDP.

Significance to Bio-Medical Research and the Program of the Institute:

Hormones act on many cells at the level of adenylate cyclase to raise cyclic AMP levels in cells. An understanding of the mechanism of nucleotide action at the adenylate cyclase regulatory site is central to solving the mechanism by which hormones transmit their message across plasma cell membranes. These studies serve to direct our attempts to dissect biochemically the adenylate cyclase system. That is, a search for a hormone-mediated GTPase is more likely to yield fruit than is an attempt to find an enzyme covalently modified by guanine nucleotide analogs.

Proposed Course of Project: Terminated.

Publications:

Reversible Activation of Hepatic Adenylate Cyclase by Guanyl-5'-YL-(α,β-methylene)diphosphonate and Guanyl-5'-YL Imidodiphosphate.; C. Londos, M.C. Lin, A.F. Welton, P.M. Lad, and M. Rodbell. J. Biol. Chem. In Press.

| SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space) | U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT** | PROJECT NUMBER ZO1 AM 15009-01 LNE |
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PERIOD COVERED

July 1, 1976 through September 31, 1977

TITLE OF PROJECT (80 characters or less)

The Mechanism of Cholera Toxin Activation of Adenylate Cyclase

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

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| OTHER: | Ann F. Welton | NIH Fellow | LNE NIAMDD |

COOPERATING UNITS (if any)

NONE

LAB/BRANCH

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The goal is to establich the exact role of guanine nucleotide in the cholera toxin activation of adenylate cyclase. By simplifying the conditions for toxin treatment of plasma membranes and by using highly purified ATP as substrate for enzyme assay, we found that the expression of activity for toxin activated adenylate cyclase has an absolute requirement for GTP. These results were interpreted by assuming that the component responsible for GTP activation is a specific GTPase and that cholera toxin treatment leads to inhibition of this GTPase, allowing GTP to activate adenylate cyclase in a manner similar to GppNHp and $GpCH_2pp$, analogs resistant to GTPase hydrolysis. This research suggests that modulation of adenylate cyclase through the GTP component may be an important mechanism for intracellular regulation of cyclic AMP levels.



PHS-6040
(Rev. 10-76)

Project Description

Objectives: Using cholera toxin as a probe, the goal of this project was to study how guanine nucleotides activate the adenylate cyclase system. Published studies suggested that cholera toxin increases cyclic AMP levels by activating adenylate cyclase and the resultant enzyme also becomes more sensitive to GTP activation. Our study was directed toward establishing the exact role of GTP in cholera toxin activation in order to further understanding of the regulation of cyclic AMP levels by guanine nucleotides.

Methods: Plasma membranes from turkey erythrocytes were used in this study for two reasons: firstly adenylate cyclase from turkey erythrocytes is more stable during treatment prior to assay for enzyme activity and secondly it is normally insensitive to GTP activation. $A_1$ peptide from cholera toxin was prepared after dissociation and alkylation of the components. Commercially available ATP was extensively purified on a DEAE-cellulose column to reduce the level of contaminating GTP. After toxin treatment, the membranes were washed before assaying adenylate cyclase activity using ATP as substrate.

Major Findings: The conditions used for treatment of erythrocyte membranes were simplified to include only membranes, NAD and $A_1$ peptide, in addition to dithiothreitol for stabilizing the enzyme activity. In contrast to plasma membranes obtained from rat liver, erythrocyte membranes were only slightly inhibited by NAD treatment. When 1 mM unpurified ATP was used for assay, toxin treatment lead to a 5 to 10-fold activation of basal activity for adenylate cyclase. However, when purified ATP was used at a concentration below 0.1 mM, activation of basal activity was decreased to less than 2-fold and only when GTP was added in the assay, was there the normally observed 10-fold activation. Moreover, GTP, which is normally a poor activator, would now activate the enzyme with the same potency as GppNHp and $GpCH_2pp$, analogs resistant to GTPase hydrolysis. These results were interpreted by assuming that the nucleotide regulatory component is a specific GTPase and that toxin treatment leads to inhibition of this GTPase, allowing FTP to activate adenylate cyclase in a manner similar to analogs resistant to GTPase hydrolysis.

Significance to Biomedical Research and the Program of the Institute: This study furthers our understanding of the mode of action of cholera toxin, a pathogen still causing wide-spread disease. Using this toxin as a tool, we now conclude that the component responsible for GTP activation plays an integral part in the regulation of adenylate cyclase. This research suggests that modulation of adenylate cyclase activity through the GTP component may be an important mechanism for intracellular regulation of cyclic AMP levels.

Proposed Course of Project: It is planned to test the effects of the cholera toxin $A_1$ subunit on other adenylate cyclase systems under conditions similar to that outlined with the erythrocyte cyclase system. However, before proceding further, we must investigate the cause of inhibition of cyclase activity by NAD observed with the hepatic system. This aspect of the in-

Proposed Course of Project (Continued):

vestigation is underway. Following demonstration of the ubiquitous actions of cholera toxin on the GTP site, studies will be carried out on the actions of the toxin on the putative GTPase site which is presumably affected by the toxin.

Publications: None

| SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space) | U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT | PROJECT NUMBER Z01 AM 15100-07 LNE |
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PERIOD COVERED
July 1, 1976 through September 31, 1977

TITLE OF PROJECT (80 characters or less)

Protein-Nucleic Acid Interactions: Chromatin Structure

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

| | | | |
|---|---|---|---|
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| OTHER: | James P. Whitlock, Jr. | Staff Fellow | LNE NIAMDD |
| | Arnold Stein | Staff Fellow | LNE NIAMDD |
| | M. Bina-Stein | Staff Fellow | LNE NIAMDD |

COOPERATING UNITS (if any)

None

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NIAMDD, NIH, Bethesda, Maryland 20014

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Extensive investigations of the structure of the chromatin core particle have been carried out by a variety of physicochemical techniques. We have determined the positions and relative susceptibilities of nuclease cleavage sites within the core particle for DNases I and II, an endonuclease from Aspergillus and micrococcal nuclease. Resistance to cleavage of the central portion of core particle DNA by those nucleases which require divalent cations likely arises from binding of the basic regions of the smaller histones to those DNA sites. The amino terminal ends of the histones appear to also bind DNA at the entry and exit points for the nucleic acid on the nucleosomes. Crosslinking experiments with core particles labeled at the 5' DNA termini suggest that H3 is located at the ends of the DNA. The role of various histones in organization of the structure of the core particle has been investigated. H3 and H4 (but not H2A and H2B) fold 140 base pair DNA into a compact structure (S = 9.2, 75 A diameter). These arginine rich histones can also fold SV40 DNA and induce torsional constraints on the DNA equivalent to those induced by all four smaller histones. A crosslinked octamer of the four smaller histones has been prepared and is used as a model for the protein core of the nucleosome.

Project Description

Objectives: To study the structural bases for the restriction of transcription of the major portion of genetic information in nucleated cells, and to study the mechanisms which allow transcription of that portion of the genetic information which is appropriate to the function of the particular cell type.

Methods Employed: Nuclei are prepared from chicken erythrocytes, rodent liver, or Hela cells, labeled with $^{3}H$-thymidine or unlabeled. Nuclear suspensions in isotonic sucrose containing 0.1 - 1 mM $CaCl_2$ are digested for varying lengths of time with micrococcal nuclease and nucleosomes isolated by sedimentation on isokinetic sucrose gradients.

Methods for chemical modification of core particles with polynucleotide kinase and radioactive ATP or with dimethyl suberimidate were as described previously.

Histones are isolated from unsheared, salt washed chromatin by modifications of published procedures involving NaCl dissociation of proteins, removal of DNA by absorption onto hydroxyapatite and fractionation of the histones by gel filtration. Reconstitution of DNA and histones is performed by salt step gradient dialysis in the absence of denaturants. SV40 DNAI and DNAIr and the relaxing extract from LA9 cells were isolated by standard procedures. Conditions for gel electrophoresis, autoradiography and physical measurements were as previously detailed.

Trypsin digestion of core particles was carried out at 20° in 5 mM Tris-Cl, pH 8.0, at a trypsin concentration of 5mcg/ml for about 2 hours. This procedure produces the same limit digest of histone fragments previously characterized by Weintraub and VanLente as consisting of the carboxyl-terminal 2/3 - 3/4 of the histones.

Electron microscopic samples were spread onto carbon coated parlodion covered copper grids pretreated with ethidium bromide. Micrographs were taken using a Philips 300 microscope operating at 40 KV. Size calibrations were made with a replica grating and length measurements obtained by tracing molecules using a Numonics, Inc. x-y digitizing stage.

Major Findings: We have previously reported the utility of 5' terminal labeling of core particle DNA followed by nuclease digestion, gel electrophoresis and autoradiography as a mapping technique for determining the positions and relative susceptibilities of cleavage sites for DNase I acting on core particle DNA. We have extended this experimental approach to map cleavage sites for other enzymes. DNase II, like DNase I, cleaves core particle DNA at ten nucleotide intervals; however, this enzyme shows no preference for any particular site. The kinetics of appearance of labeled fragments in a DNase II digest looks as if the enzyme randomly cleaves any of the 13 potential cleavage sites within the nucleosome. In contrast, an endonuclease isolated from Aspergillus oxyzae cleaves core particle DNA in a fashion highly similar to that observed for DNase I. Since the two enzymes which show restricted cleavage

Major Findings (Continued)

at sites 30, 60-8-, and 110 nucleotides from the 5' end of DNA both require divalent cations, we suggest that these sites may be bound by histones in a fashion which precludes formation of a catalytically active ternary complex of substrate, enzyme and divalent cation.

Micrococcal nuclease also produces a characteristic pattern of stained DNA fragments on digestion of core particle DNA. In contrast to the other nucleases, which make single stranded nicks at ten nucleotide intervals along the DNA, this latter enzyme makes primary scissions of core particle DNA at sites 21, 32, 53, 102 and 115 bases from the ends. The nicking endonucleases prefer double stranded DNA as substrate, micrococcal nuclease, on the other hand, prefers a single stranded substrate. Thus, it seems possible that the two classes of nucleases may recognize different features of the structure of DNA within the core particle leading to different primary cleavage sites.

All three of the enzymes which fail to readily hydrolyze the central portion (60-80 nucleotides from the ends) of core particle DNA require divalent cations for activity. We have examined the possibility that the protection of this region of DNA from these hydrolases might arise from binding of histones to the nucleic acid at these sites by selective tryptic proteolysis of core particles, removing the highly basic amino terminal regions of the histones from the complex. Amino acid analyses of trypsin-treated core particles are consistent with the notion that the protease excises the amino terminal regions of the four smaller histones. Following trypsin treatment, the particle remains compact with a sedimentation coefficient of 9.2 S; DNA folding is further demonstrated by the relatively small change in the circular dichroism spectrum of the particle after removal of the histone amino terminal regions. Nicking sites at ten base intervals persist in the trypsin treated core particle when digested with DNase I. Strikingly, however, the sites 60-80 nucleotides from the ends, and to a lesser extent the site 30 nucleotides from the end, resistant to cleavage in the intact core particle, become highly accessible to the nuclease after trypsin treatment. We therefore operationally define these loci as likely sites of interaction of the amino terminal regions of the smaller histones with the DNA of the nucleosome.

The ends of the DNA in the core particle are also relatively resistant to nucleases, since the particle is produced by micrococcal nuclease degradation of chromatin. Trypsin digestion of core particles prior to micrococcal nuclease digestion leads to a marked increase in the rate of digestion of the terminal label with a smaller increase in rate of digestion of internal DNA. Thus, the sites at the ends of the core particle DNA also appear to interact with the highly basic amino terminal regions of the histones.

To investigate which histones interact with the ends of core particle DNA, we have utilized a DNA-protein crosslinking procedure introduced by Levina and Mirzabekov, applying it to core particles labeled at the 5' DNA termini. Initial results obtained with this procedure were complicated in interpretation by the presence in our Hela core particle preparations of an active protein

Major Findings (Continued)

kinase. We have, therefore, removed the kinase from particle preparations by salt washing. Using these particles, the level of modification of histones due to the protein Kinase is reduced 50-100 fold compared to unwashed particles. Most of the DNA label which can be crosslinked to histones is associated with histone H3; however, the efficiency of the crosslinking is low (about 5% of the theoretical maximum). Whether this reflects the multistep nature of the procedure or whether only a fraction of the particles have H3 at the ends and the remainder have another histone at the end, but not with a sufficiently proximate lysyl residue to allow crosslinking, is currently uncertain.

Association of one of the arginine rich histones with the ends of core particle DNA is very consistent with another set of experiments we have performed, experiments which indicate the critical role of these histones, H3 and H4, in folding and compaction of the DNA in core particle structures. Association of H3 + H4 with 140 base pair DNA, followed by sucrose gradient centrifugation, allows the isolation of a compact particle containing a tetramer of these histones associated with the nucleic acid. The particle has a sedimentation coefficient of 9.8 S and appears in the electron microscope as a 75 A diameter spherical particle. Several of the morphological features characteristic of the native core particle are faithfully present in the partial core particle formed with the arginine rich histones alone. H3 + H4 can also organize longer pieces of DNA. Complexes of these histones with SV40 DNAI are relaxed in electron micrographs and compacted 2.6 fold. About 17 spherical particles are present along the DNA in such complexes. The number of particles observed for fully relaxed complexes and the length compaction allow us to calculate that about 160 base pairs of DNA are associated with H3 + H4 alone in a particle while all four smaller histones organize about 170 base pairs. The relaxed appearance of such compleses suggests that H3 + H4 can induce torsional constraints on superhelical DNA. This suggestion was directly examined by association of H3 + H4 with either SV40 DNAI or DNAIr, treatment of the resultant complexes with relaxing extract, deproteinization, and examination of the topological state of the resultant DNA by agarose gel electrophoresis. Addition of the arginine rich histones induces torsional alterations on either superhelical or relaxed closed circular DNA. The degree of alteration of topological state appears to be about the same for given molar ratios of the arginine rich histones, on the one hand, or all four smaller histones, on the other. Thus, these proteins appear to form the primary structural framework for folding and compaction of DNA within the nucleosome.

Other features of core particle structure have been elucidated through study of the interactions of DNA with a crosslinked octamer of the four smaller histones. Monomer core particles are crosslinked with dimethyl suberimidate, reisolated and their physical propertied compared with native core particles. As might be expected from our previous studies of monofunctional amidination of the lysyl residues of histones, little perfurbation of nucleosome structure results from substitution of about 75% of these basic residues by the crosslinking agent. DNA can be removed from the proteins by simple addition of NaCl to 3M, leaving a crosslinked, pure, octameric complex of the histones. This complex protein sediments at 5.3 S and recombines with 140 base pair DNA to form a particle identical to the crosslinked core particle from which it was isolated. Both DNA removal and reassociation occur in the total absence

Major Findings (Continued)
of denaturants. The histone octamer also compacts SV40 DNAI and induces torsional constraints on both relaxed and superhelical closed circular viral DNA molecules. Studies such as these demonstrate that DNA is outside the histone core in the nucleosome and suggest that energy derived from histone-histone interactions is not required for the folding of DNA within the core particle. This protein will be used as a model for the protein nucleus of the core particle in future studies of the association of DNA and histones in chromatin.

Significance to Bio-Medical Research and the Program of the Institute
Description of the structure of the eukaryotic nucleoprotein complex, chromatin, is necessary for understanding the mechanism of gene regulation in higher organisms. Our current studies aim at complete understanding of the structural relationships between histones and DNA to create the nucleosome, the basic structure determining element of chromatin.

Proposed Course of Project: Investigations of histone-histone interactions and the mechanism of stabilization of DNA by its interaction with nucleosome cores will continue using techniques outlined above.

Publications:

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Publications Continued:

Whitlock, J.P. Jr. (1977). Staphylococcal nuclease and pancreatic DNase cleave the DNA within the chromatin core particle at different sites. J. Biol. Chem., in press.

Whitlock, J.P. Jr. and Simpson, R.T. (1976) Removal of histone H1 exposes a fifty base pair DNA segment between nucleosomes. Biochemistry 15: 3307-3314.

Whitlock, J.P. Jr. and Simpson, R.T.;Preparation and physical characterization of a homogeneous population of monomeric nucleosomes from HeLa cells. Nucleic Acids Res. 3: 2255-2266.

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Whitlock, J.P. Jr., Rushizky, G.W. and Simpson, R.T. (1977). DNase-sensitive sites in nucleosomes. Their relative susceptibilities depend on nuclease used. J. Biol. Chem. 252: 3003-3006.

| SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space) | U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT | PROJECT NUMBER ZO1 AM 15101-06 LNE |
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PERIOD COVERED
July 1, 1976 through September 30, 1977

TITLE OF PROJECT (80 characters or less)

Analysis of the Active and Inactive Portions of the Eukaryotic Genome

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

| | | | |
|---|---|---|---|
| PI: | Robert T. Simpson | Sr. Surgeon, (R) (P) | LNE NIAMDD |
| | Michael Bustin | Visiting Scientist | LNE NIAMDD |
| OTHER: | Donald Gruol | Staff Fellow | LNE NIAMDD |
| | Sunao Yamazaki | Int. Fellow | LNE NIAMDD |
| | William Rizzo | Guest Fellow | LNE NIAMDD |
| | Steve McKnight | Grad. Student | Dept. of Biology, Univ. of Virginia |
| | Oscar Miller | Professor | " " |
| | Paul Kurth | Grad. Student | Dept. of Biology, Johns |
| | E.N. Moudrianiakis | Professor | Hopkins University |
| | Ronald Reeder | Staff Member | Carnegie Institution |

COOPERATING UNITS (if any)
Department of Biology, University of Virginia
Department of Biology, Johns Hopkins University
Carnegie Institution of Washington, Department of Embryology

LAB/BRANCH
Laboratory of Nutrition and Endocrinology

SECTION
Developmental Biochemistry Section

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20014

| TOTAL MANYEARS: | PROFESSIONAL: | OTHER: |
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CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES XXX(c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Antibodies to various isolated histone classes demonstrate the association of histones with transcriptionally active nonribosomal genes in embryos of Drosophila melanogaster and the depletion of histones in the puffed regions of the polytene Chromosomes of Chironomous thummi. The staining of histones in puffs is markedly reduced when compared to adjacent bands or interbands. Newly replicated chromatin is associated with an unaltered complement of histones as detected immunologically. Concanavalin A, a plant lectin, binds specifically to chromatin from rat liver with a high association constant. Electrophoresis followed by detection of proteins by their binding of the lectin reveals that a limited number of the nonhistone proteins have sugar residues bound by concanavalin A. The lectin thus might serve as a nondisruptive probe for the chromosomal glycoproteins. A probe different in kind, triiodothyronine, also binds specifically and with high affinity to liver chromatin. In vivo experiments suggest a physiologic relevance for the class of binding sites under study. Using the binding assay previously described, certain additional features of the interaction have been discerned; of particularly interest is an apparent Cooperativity for the interactions of the hormone with chromatin.

Project Description

Objectives: To investigate the physical and chemical differences between portions of the eukaryotic genome which are transcribed and those which are repressed.

Methods Employed: Histones, isolated from calf thymus, are fractionated by established methods and then complexed with RNA. Antibodies to these complexes are elicited in rabbits. When necessary, antibodies to individual histones are purified by affinity chromatography on histone-Sepharose columns. Antibody titers are assayed by the microcomplement fixation technique.

Microscopic studies were performed by standard procedures, using phase contrast and fluoresence microscopy for the chironomous system and electron microscopy of Miller spreads for Drosophila embryos. Detection of antibody in both cases was by sandwich techniques, using fluorescing or ferritin labeled anti-rabbit IgG antibodies.

Concanavalin A was used either as a tritium labeled acetylated preparation or iodinated with radioactive iodine. Binding assays were conducted at various concentrations of lectin and chromatin; the extent of binding was determined by addition of NaCl to 0.15 M and centrifugation of the chromatin to pellet it away from unbound lectin. Concanavalin A binding proteins on gel electrophoretic slabs were detected by incubation with iodinated lectin, washing to remove unbound material and autoradiography.

Chromatin preparations were as previously described except that all chromatin samples utilized for lectin binding studies were centrifuged through 1.7 M sucrose containing 0.1% Triton X-100 to ensure removal of nuclear membrane contaminants. Preparations of core particles were made as previously detailed.

Isolated chromatin, chromatin fractions, or nucleosomes are incubated at various concentrations and ionic conditions with $^{125}$I-triiodothyronine, T3. Temperature and time of incubation are varied. Replicate tubes contain the same constituents plus a 200-fold excess of unlabeled T3 as a measure of nonspecific binding of the hormone. At the conclusion of each incubation, a buffer containing 0.5% Tritium X-100 is added along with a measured quantity of hydroxyapatite. The tubes are centrifuged and washed several times with buffer. The final hydroxyapatite pellet is counted in a gamma spectrometer.

Major Findings: A major problem in the use of antibodies to probe the structure and organization of chromatin is the availability of purified histone fractions to elicit the antibodies. Using microcomplement fixation techniques, we have been able to demonstrate that for the four smaller histones, H2A, H2B, H3, and H4, a high degree of crossreactivity exists for histones derived from calf and Drosophila. Coupled with the observations of others, this suggests that antibodies to readily available calf histones can serve as universal reagents for the study of the organization of histones in a wide variety of tissues. This allows studies on organisms where purification of sufficient

Major Findings (Continued)

amounts of histones for antibody elicitation is difficult, ensures that the immunological reactions detected arise from cross-reacting histone determinants rather than species specific nonhistone determinants, and facilitates comparison of results from various investigations.

Using these antibodies, we have investigated the structural organization of chromatin in two systems which are ideally suited for molecular level study of chromatin transcription. The polytene chromosomes of Chironomus thummi allow resolution of individual genetic units at the level of the light microscope. Antibodies to all four smaller histones stain both the bands and interbands of Chironomous polytene chromosomes equivalently. Staining is proportional to the density of the band in the phase contrast microscope. In contrast, while antibodies stain the three permanently puffed regions of chromosome IV (puffs are thought to be regions of high transcriptional activity), the degree of staining is less than observed for adjacent bands or interbands. Similar results are obtained for staining of heat shock induced puffs by anti H3 + H4 in Drosophila melanogaster. In this latter case, antibody staining with anti-RNA polymerase demonstrates that there is no absolute steric barrier to the interaction of antibody with chromatin regions. Currently, in view of these and biochemical data, we feel it likely that the histones are present in regions of transcriptional activity, but that their accessability to specific antibodies may well be partially masked by other proteins associated with this active region of the genome.

This conclusion has been strengthened by studies at the electron microscopic level with specific antihistone antibodies. The preblastoderm embryo of Drosophila melanogaster provides a nearly unique tissue source, having active transcription of nonribosomal genes and extremely active replication of DNA. Miller spreads of such neclei show the presence of numerous transcription units and replication eye bubbles. Interestingly, statistical analysis of the distribution of histones along both types of metabolically active chromatin, compared with nonreplicating, transcriptionally inactive chromatin, indicates no significant differences, when studied with antihistone antibodies and ferritin labeled anti-rabbit IgG. The presence of histones on transcriptionally active chromatin is not inconsistent with biochemical evidence available at this time. The presence of histones on both branches of a replication fork is at variance with the nuclease digestibility of replicating chromatin - this difference may be related to the extremely rapid S phase in Drosophila embryos.

The above studies demonstrate the utility of in situ studies for evaluation of the organization of histones in a variety of functional states of chrmoatin. Similar probes for study of the nonhistone components of chromatin have been difficult to obtain. The observation that the plant lectin, concanavalin A (Con A) bound specifically to nuclei suggested that this molecule might serve as such a probe. We have demonstrated that the lectin binds specifically to rat liver chromatin with a large association constant ($<10^7$M). The binding has appropriate association and dissociation kinetics for this stability of interaction; Scatchard analysis indicates the presence of a single class of binding sites. Gel electrophoretic separation of the proteins

Major Findings (Continued)

of chromatin demonstrates that three components bind the bulk of Con A associated with chromosomal proteins. While the potential structural or functional roles of these three proteins are currently unknown, Con A should provide a useful probe into their organization in chromatin at various levels, enabling a beginning of $\chi$ studies on nonhistone chromosomal components analogous to those described above for the histones.

In addition to probes for histones and unidentified nonhistones in their interactions with chromatin, we are studying the interactions of a physiologically relevant small molecular probe with the eukaryotic genome. Triiodothyronine (T3) induces a pleotropic set of effects on the function of a variety of animal cells. Previous studies have suggested specific binding of this hormone to nuclei and chromatin and, using a newly devised method of assessment of binding, we have confirmed this notion. A part of our interest in this probe derives from the observation of others that, in contrast to steroid hormones, no cytoplasmic receptor appears to be involved in the interactions of T3 with chromatin.

Under appropriate conditions, T3 binds to the chromatin of thyroidectomized rat liver with an association constant of about $10^{11}$ $M^{-1}$. The number of sites involved in binding at this level of stability is about per haploid genomic equivalent, far smaller than the numbers reported for other methods of assessment of T3 binding or those studied for steroid hormones. This limited number of binding sites suggests the physiologic relevance of the detected interaction. This latter idea is supported by studies which demonstrate a reduced number of T3 binding sites in normal (vs. thyroidectomized) rats and the saturation of these sites by in vivo administration of T3 prior to isolation of liver chromatin from thyroidectomized animals.

In contrast to the kinetics of interaction of small regulatory molecules with their genomic protein receptors for prokaryotic systems (e.g. IPTG and the lac repressor), the interactions of T3 with chromatin appear to have cooperative kinetics. Hill plots of the interaction have a coefficient of 1.6-2, suggesting a dimer of the thyroid hormone receptor protein is the minimal functional species. Analysis of Scatchard plots for the binding are quite consistent with this interpretation.

Significance to Bio-Medical Research and the Program of the Institute.
One of the most direct approaches to understanding the gross basis for transcriptional regulation of eukaryotic cells is study of the composition and structure of portions of the genome which are active as transcriptional templates and comparison with properties of repressed segments. In the past we have detailed the propertied of segments of chromatin which are active as templates for RNA polymerase *in vitro* and compared them with inactive chromatin. During the past year we have concentrated on the development of a series of probes which more accurately reflect the in situ organization of metabolically active portions of the chromosome. The use of the variety of probes, coupled with the various systems that we are utilizing for assay, seems likely to produce additional significant information about the organization of biologically interesting segments of chromatin. Our eventual goal is description of the mechanism of transcriptional regulation in animal cells -

Significance to Bio-Medical Research and the Program of the Institute (Cont.)

the features responsible for the stable differentiated state of a normal mature cell. Understanding of these features will facilitate understanding of neoplastic transformation and normal development and differentiation.

Proposed Course of Project: Further definition of the organization of histones in both ribosomal and nonribosomal genes will be obtained, using antihitone antibodies. We will attempt to define the organization of histone H1 in the various systems which we have studied. The receptor protein for Con A will be isolated and its enzymatic properties (if any) studied and its structural effects on chromatin ascertained. We will continue to study the interactions of T3 with chromatin, localizing its binding to proteins or DNA and attempting to isolate putative regulatory regions by affinity chromatographic methods.

Publications:

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Bustin, M., Kurth, P.D., Moudrianakis, E.N., Goldblatt, D., Sperling, R. and Rizzo, W. (1977) Immunological probes for chromatin structure. Cold Spring Harbor Symp. Quant. Biol., in press.

Bustin, M., Reeder, R. and McKnight, S.L. (1977) Immunological cross-reaction between calf and Drosophila histones. J. Biol. Chem. 252:3099-3101.

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Simpson R.T. Chromatin fractionation by chromatography on ECTHAM-cellulose. In Stein, G., Stein, J. and Kleinsmith, L. (Eds.): Methods in Chromosomal Protein Research. Academic Press, New York, in press.

| SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space) | U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT** | PROJECT NUMBER Z01 AM 15102-17 LNE |
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PERIOD COVERED
July 1, 1976 through September 31, 1977

TITLE OF PROJECT (80 characters or less)

Study of Ribonuclease (barnase) and its Inhibitor (barstar) from Bacillus amyloliquefaciens.

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: Robert W. Hartley Research Chemist LNE/NIAMDD

OTHER: None

COOPERATING UNITS (if any)
Large-scale Laboratory, Office of Chief LNE-NIAMDD; Collaboration with D.M. Blow, Department of Biophysics, Imperial College, London, and with G. Bricogne and R.S. Sheppard, Lab. of Molecular Biology, MRC, Cambridge

LAB/BRANCH
Laboratory of Nutrition and Endocrinology

SECTION
Developmental Biochemistry Section

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland, 20014

| TOTAL MANYEARS: | PROFESSIONAL: | OTHER: |
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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Two proteins, barnase, the extracellular ribonuclease of Bacillus amyloliquefaciens, and barstar, its intracellular inhibitor, are being developed as a model system for the study of protein folding and Protein-protein interactions. Amino acid sequence determination and X-ray structure characterization are being carried out along with thermodynamic studies of folding reactions. A peptide complementation system for barnas has been developed which will be used to investigate the roles of specific residues in both folding and enzymatic activity.

Project Description

Objectives: To further characterize and investigate this enzyme and its natural inhibitor by physical, chemical, and biological techniques. To develop methods of culture, genetic manipulation and genetic analysis so that the organism and its product enzyme-inhibitor pair may be applied to a broad study of structure, function and synthesis.

Specific objectives are: 1) the determination of the amino acid sequence and three-dimensional structure (by X-ray diffraction) of both proteins and the structure of their complex; 2) thermodynamic and kinetic analysis of structural transitions produced in both proteins by heat or denaturing reagents; 3) analysis of the folding and activity of both by means of peptide complementation systems analogous to those being used for the study of pancreatic ribonuclease and micrococcal nuclease; 4) all of the above with chemically and/or genetically modified sequences.

Methods Employed: B. amyloliquefaciens (strain H and derivatives and several others) are grown in synthetic media. Barnase is absorbed from the culture medium onto phosphocellulose, Barstar is extracted from the cells. Their purifications make use of ion exchange, gel-permeation and affinity chromatography.

Work on the determination of amino-acid sequences involves amino acid analysis, enzymatic digestions, peptide separations and peptide sequencing by dansyl-Edman and difference-Edman techniques. Peptide separation and amino acid analysis are by automated ion-exchange chromatography using a modified JEOL analyzer equipped for detection with Roth's orthophthaldehyde reagent. Dansyl amino acids are identified by thin-layer chromatography on polyamide layers.

The reversible transitions undergone by barnase and barstar and their derivatives under the influence of temperature changes or denaturing agents are studied primarily by ultraviolet and fluorescence spectroscopy, but also by optical rotatory dispersion, circular dichroism, exclusion chromatography with the use of proteolytic enzymes and by equilibrium binding studies. These techniques are also used to study complementation reactions between natural and synthetic peptide sequences of the two proteins as well as conformational properties of each peptide alone. Studies of the peptide derivatives also include use of antibodies, chromatography, crystal growth, etc.

Crystals are grown in hanging drops (10 µl) suspended over suitable solvents. Isomorphous crystals containing heavy atoms are prepared by soaking crystals in solutions of appropriate heavy atom compounds. The three dimensional structure of barnase is being determined by methods of X-ray crystallography.

The Merrifield procedure has been used to synthesize portions of the barnase sequence.

Major Findings: Complementation of barnase (1-102) by several peptides from the C-terminal region has been studied further. Peptide (88-110) yields up to 60% activity with a dissociation coefficient of $8 \times 10^{-6}$ M. The same peptide with all of its amino groups blocked by succinylation binds about as well and yields an activity of about 10%, indicating that neither Lys-98 or Lys-108 is directly involved in the active site. Peptide (95-110) again binds about as well, but with a limit activity of 20%, showing that the stretch from Arz-83 to Len 95 contributes little to the binding and does not participate directly in the active site. Peptides - (98-108) and - (103-110) do not complement bar(1-102), nor do they bind strongly enough to inhibit complementation by peptide (88-110).

It has been found that exposure of barnase crystals (in high salt) to $KI_3$ results rapidly in the iodination of Tyr-17 to diiodotyrosine and possibly of Tyr-24 and His-18. Neither His-102 nor any of the other 5 tyrosines is affected, even after long (16 hr.) exposure. The iodinated barnase retains full activity and the morphology of its crystals is not visibly altered. Iodination in solution (in low salt) is similar, but is followed in a short time (<1 hr.) by further tyrosine modification and loss of activity.

Significance to Bio-Medical Research and the Program of the Institute. The small size, stability, easily measured enzyme activity and absence of disulfide bridges in barnase make it an excellent choice for a long-range study of the relation between the sequence of an enzyme and its structure and function. The inhibitor is an even simpler protein and should be useful in a similar fashion. Together, they offer a model system for the study of protein-protein interaction.

The nature and extent of the thermal transition of barnase gives strong support to the concept that the three-dimensional structure of a protein may be completely determined by its amino acid sequence. The simplicity and all-or-none nature of the transition should make it useful for thermodynamic analysis of the factors involved in maintaining a folded protein structure. The production of both of these proteins by the same organism is also of biological interest.

The specific iodination of one or more residues without inactivation or change in crystal morphology may yield a good heavy-atom isomorphous replacement, providing the Key we need to solve the barnase structure by X-ray diffraction. The possible modification of Hl-18 is interesting in that it is known that at least one of the two histodines is involved in the active site, and one also has an abnormally high pK, as revealed by NMR studies.

Proposed Course of Project: Collaboration with the workers in London and Cambridge on the X-ray structure will continue. Further characterization of the iodinated barnase will be carried out. The complementation work will continue, with emphasis on the effects of modification of specific residues.

Publications:

Hartley, R.W.: Complementation of Peptides of barnase, the Extracellular Ribonuclease at Bacillus amyloliquefaciens. J. Biol. Chem. 252, 3252,(1977).

| SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space) | U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT** | PROJECT NUMBER ZO1 AM 15104-05 LNE |
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PERIOD COVERED
July 1, 1976 through September 31, 1977

TITLE OF PROJECT (80 characters or less)

Preparation of Large Oligonucleotides from Purified Nucleic Acids.

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

| | | | |
|---|---|---|---|
| PI: | George Rushizky | Research Chemist | LNE/NIAMDD |
| OTHER: | David Rogerson | Head, Pilot Plant | LNE NIAMDD |

COOPERATING UNITS (if any)

Large-scale laboratory, Pilot Plant, LNE, NIAMDD

LAB/BRANCH
Laboratory of Nutrition and Endocrinology

SECTION

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20014

| TOTAL MANYEARS: | PROFESSIONAL: | OTHER: |
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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Hydrolysis of nucleic acids with nucleases is used for the preparation of large oligomers suitable for studies of their chemical, enzymological, and biological properties.



PHS-6040
(Rev. 10-76)

Project Description

Objectives: To isolate and/or develop enzymatic procedures for the preparation and isolation of large oligonucleotides in preparative amounts, using in part the facilities of the Pilot Plant, LNE. The oligomers will be used for chemical, enzymological and biological studies.

Methods Employed: Phages, bacteria and eukaryotic organisms were grown up for isolation of their DNAs. The latter were hydrolyzed by nuclease isolated in the lab or available commercially, and the products characterized by gel electrophoresis and standard chromatographic procedure.

Major Findings: Bacteria were grown up in the pilot plant, and Restriction enzyme purified by different procedures. Of 11 enzyme sources examined 7 enzymes (ALVI, Hpa I, BGL I and II, XNO I and II, and BLU I) were obtained in useful concentrations. Failure to isolate the other enzymes was due to various factors, ranging from variable sensitivity to sonication to apparent absence of the enzymes before purification. A survey of physical properties such as mol. wt., pI, metal-salt-, and SH cpd. activation was undertaken. The assay was by strip chart counting of $^{32}P$ T7 DNA digests fractionated by gel electrophoresis. The survey is not completed.

Several R enzymes (Bam I, Bgl I and Bgl II) were used to prepare T7 DNA digests for studies on preparative scale fractionation by column chromotography. Best results were with the HA X8 (Hamilton Co.) ion exchanger, reported to give better fractionation of tRNAs than RPCS. This is said to be due to differences in the exchanges; HA X8 consists of a porous 8% crosslinked vinyl benzene lattice in uniform bead ( 20 ± 5 μm) form, while RPCS consists of teflon chips coated with a quatenary $NH_3$ derivative more hydrophobic than vinyl benzene. Fractionation of R enzyme fragments on RPCS shows extended peaks without much detail, analysis of which revealed separation by other criteria not by size alone. With HA X8, more detail was seen in the peaks, but good separations were obtained only with DNAs, cleaved in a few places only.

A new endonuclease was isolated from Aspergillus that cleaves double-stranded DNA to 5'-phosphate terminal compounds. The enzyme was purified 1550-fold in 25% yield by standard procedures, free of contaminating nucleases. It was characterized with Dr. J. P. Whitlock with respect to properties such as mol. wt., pI, and enzymatic specificity.

Digest of calf thymus and E. coli DNA yielded oligomers of chainlengths ranging from 10-200, with smaller compounds detected only when large (100mg) amounts of DNA digests were fractionated by column chromatography on DEAE-Sephadex A-25 in 1 M urea.

The Aspergillus DNase was used for several purposes. Hydrolysis of the chromatin subunit allowed comparison of the influence of physicochemical properties of different DNases (DNase I and II) on their interaction with nucleoprotein substrates (Whitlock et al., 1977).

The Aspergillus DNase was also employed to generate small double-stranded oligomers of chainlength 10 and higher, which are of interest for studies on nucleosome structure. This proved to be a difficult problem due to instability of the oligomers unless kept in high salt or $Mg^{+2}$ which in turn hindered their isolation and fractionation, and decreased the enzyme activity itself. Also, while the Aspergillus DNase produces square cuts in DNA (cuts across both DNA strands at the same position) at the beginning of hydrolysis as measured by viscometry, it produces compounds with single-stranded tails or loops during later stages of hydrolysis.

Two procedures were employed to avoid these problems in the preparation of small oligomers. First, DNA was hydrolyzed with the DNase, then with $S_1$ nuclease, and the digests fractionated by stepwise elution (from 0.2 to M NaCl) from DEAE-cellulose. The compounds so obtained were resistant to further $S_1$ nuclease hydrolysis unless treated with HCHO, and melted (carried out by Dr. A. Stein) like double-stranded oligomers at the expected temperatures. Chainlength assignments by denaturing and non-denaturing gel electrophoresis gave a range of 10-60 for the fraction eluted by M NaCl (after washing with 0.2 M NaCl.). As an alternative procedure, DNase digests were heat-denatured, reannealed at 70°, and the highly repetitive DNA fraction melting between 70° and 96° isolated on calcium phosphate. After desalting and $S_1$ nuclease hydrolysis, fractionation was on DEAE-cellulose. This project is not completed.

Three simple eukaryotes were examined for ease of growth, and isolation of DNA. Dictyostelium discoideum was difficult to grow in large amounts, and Aspergillus, while readily grown, proved to be difficult to lyse. Euglena were obtained in good yields ( with Dr. D. Groul and E. Kempner) and DNA prepared with mol. wt. comparable to λ DNA (about $25 \times 10^6$).

Work with $S_1$ nuclease on tRNA has been concluded.

Significance to Bio-Medical Research and the Program of the Institute. The R-enzymes purified will be used for studies on eukaryotic DNAs. Availability of double-stranded oligomers in purified form will facilitate projects, also in collaboration with others, that require such compounds in preparative amounts.

Procedures for the isolation of highly repetitive DNA fragments from eukaryotes further the study of their structure and possible function.

While Euglena has no mutants or viruses and highly polyploid, it represents a source of eukaryotic DNA that is readily prepared in mg amounts. The cells are maybe labeled, and procedures for the isolation of DNA- and RNA polyerase have been described.

Proposed Course of Project: The long-range purpose involves the understanding of the genome arrangement of eukaryotic organisms. This involves enzymatic degradation of DNA to fragments for characterization of their structure and function properties. To begin with, highly repetitive DNA and palindrome will be studied.

Publications:

Rushizky, G.W. and Mozejko, J.H., Optimization of Conditions for Cleavage of tRNA at the Anticodon loop by $S_1$ nuclease; Analytical Biochemistry 77, 562-566, 1977.

Rushizky, G.W. and J.P. Whitlock, Jr.; Preparation and Properties of a New DNase from Aspergillus oxyzae.

Biochemistry, in press.

| SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space) | U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT | PROJECT NUMBER ZO1 AM 15105-01 LNE |
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PERIOD COVERED
July 1, 1976 through September 31, 1977

TITLE OF PROJECT (80 characters or less)

A Developmental Transition in Mosses as a model for the study of Eukaryotic Genome Control

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: Robert W. Hartley Research Chemist LNE/NIAMDD

OTHER: None

COOPERATING UNITS (if any)

NONE

LAB/BRANCH
Laboratory of Nutrition and Endocrinology

SECTION
Developmental Biochemistry Section

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20014

| TOTAL MANYEARS: | PROFESSIONAL: | OTHER: |
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| 0.3 | 0.2 | 0.1 |

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The developmental transition of the moss gametophyte from filamentous protonema to leafy gametophore is being developed as a model for eukaryotic systems in which large blocks of genes are switched on or off the transition (in Nanomitrium austinii) may be induced by cytokinins or, reversibly, by changes in temperature.

Project Description

Objectives: In their development, mosses pass through two distinct generations, represented by the haploid gametophyte and the diploid sporophyte. The haploid generation is further divided into at least two distinct morphological stages, the mature "leafy" gametophore being preceded by the purely filamentous protonema. The transition between these two forms may, in some cases, be controlled by temperature or by plant growth substances of the cytokinin (6-N substituted aminopurine) type. The current objective is to bring into aseptic culture one or several moss species and study this discrete transition as an instance of coordinated gene-switching in a typical eukaryote.

Methods Employed: Asceptic cultures are obtained by spreading the contents of surface-sterilized spore capsules onto suitable agar media. Culture thereafter is largely by standard microbiological techniques.

Major Findings: While a number of mosses have been brought into culture, work so far has concentrated on Nanomitrium austinii (Ephemoraceae), a rapidly developing annual moss that normally completes its development within a few weeks on the mud shores of temporarily lowered lakes or streams. At 25° it grows indefinitely (at least 8 months) as the filamentous protonemal form, on agar or semipermiable membrane surface or in deep culture. Within a week after the temperature is lowered to 15°, leafy gametophytes form and sexual development ensues, proceeding through to diploid sporophyte generation and completing its cycle with the production of spores. Raising the temperature before the sporophytes appear results in developmental reversal, protonemal filaments growing out all over the leafy gametophores.

Application of Kinitin (6-furfurylaminopurine) at $10^{-7}$ M brings on the production of normal looking gamatophores within 2 or 3 days at 25°, but sexual development at this temperature has not been obtained.

Significance to Biomedical Research and the Program of the Institute
The moss protonema-gametophore transition may offer a convenient system for approaching the general problem of gene control in eukaryotes, particularly in the developmental aspect where large sections of the genome are turned on or off.

Proposed Course of Project: The biochemical fate of Kinetin and other cytokinins will be investigated including a search for specific receptors. It is hoped that various types of developmental mutants can be isolated and genetic analysis of these can be applied to the problem.

Publications: NONE

| SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space) | U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT** | PROJECT NUMBER Z01 AM 15200-17 |
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PERIOD COVERED

July 1, 1976 through September 31, 1977

TITLE OF PROJECT (80 characters or less)

Studies on Folic Acid

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

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| PI: | Dr. Bernard T. Kaufman | Research Chemist | LNE NIAMDD |
| | Dr. Verne Kemerer | Staff Fellow | LNE NIAMDD |
| | Dr. Elizabeth Barbehenn | Staff Fellow | LNE NIAMDD |
| OTHER: | Ms. R.C. Gardiner and Ms. M.K. Romine | | |

COOPERATING UNITS (if any)

NONE

LAB/BRANCH

Laboratory of Nutrition and Endocrinology

SECTION

Vitamin Metabolism Section

INSTITUTE AND LOCATION

NIAMDD, NIH, Bethesda, Maryland 20014

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| 4.0 | 2.0 | 2.0 |

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Chicken liver dihydrofolate reductase is shown to contain a single cysteine residue and thus a single sulfhydryl group. This functional group is found to be readily titrated by both p-hydroxymercuribenzoate and dithionitrobenzoate although the dithionitrobenzoate reacts much more slowly than the organic mercurial. The binding of p-hydroxymercuribenzoate as well as a series of other organic mercurials such as methylmercuric hydroxide results in a marked stimulation of the reductase reaction. In contrast, the binding of thionitrobenzolate resulte in inhibition of activity. Evidence is presented that suggests that this sulfhydryl group is not at the active site of the enzyme since the interaction with the mercurials is not affected by the presence of a large excess of substrate. The results suggest that binding of a specific substance to this sulfhydryl group results in a conformational change in the protein yielding a more active enzymatic species. However attempts to demonstrate such a conformational change by a variety of methods have not been successful. Thus the effect on the tertiary structure of the protein must be very small. Kinetic studies suggest that the end result of this change may be related to the binding of the substrates.

Project Description

Objectives: Dihydrofolate reductase, an enzyme which is concerned with the conversion of the essential - folic acid - to the biologically active co-factor - tetrahydrofolic acid, continues to be the subject of intensive investigation because of its central role in cellular proliferation. In addition, dihydrofolate reductase is believed to be the primary target enzyme for on of the most effective cancer chemotherapeutic drugs, the anti-vitamin, methotrexate. For these reasons, we have isolated and purified to homogeneity dihydrofolate reductase from two vertibrate livers, beef and chicken. Using these highly purified materials, we have determined for the first time many of the molecular parameters of these animal enzymes. Thus, it is the purpose of this project to continue with a more detailed analysis of the animal dihydrofolate reductases in order to gain more detailed insight at the molecular level of the interaction of the enzyme with both its natural substrates and chemotherapeutic agents.

Methods Employed: Affinity chromatography using a chromatographic system based on the drug methotrexate coupled to sepharose has been used to obtain dihydrofolic reductase from liver extracts. Further purification and characterization involved the use of isoelectric focusing. Additional methods of chemical, enzymatic, and physical analysis involved kinetic measurements by spectrophotometry, molecular weights and hydrodynamics by ultracentrifugation, and molecular conformation by circular dichroism.

Major Findings: Amino acid analysis shows that chicken liver dihydrofolate reductase contains only one cysteine residue rather than two as previously reported in the literature. This observation is confirmed by the direct titration for sulfhydryl content with p-hydroxymercuribenzoate (pHMB) and dithionitrobenzoate (DTNB). Each titration, with or without 6 M guanidine hydrochloride, shows the presence of one sulfhydryl group. Thus no disulfide bonds can exist in chicken liver dihydrofolate reductase as has been postulated. The reaction with pHMB is very rapid and quantative. This suggests that this sulfhydryl group is exposed and quite reactive. On the other hand, the reaction with DTNB is significantly slower which in turn suggests that the sulfydryl group is surrounded by a hydrophobic environment which could account for the lower reactivity with the more polar DTNB.

As was previously reported, treatment of chicken liver dihydrofolate reductase with pHMB results in a marked stimulation of the reductase reaction. This current study demonstrates that it is the binding of one equivalent of this organic mercurial to the single sulfhydryl group that results in a 7-fold increase in the reactivity of the enzyme. All organic mercurials examined appear to activate the enzyme in a similar manner, i.e. binding to a single sulfhydryl group. The degree of activation appears to depend on the complexity of the organic residue. Thus the simplest mercurial, methylmercuric hydroxide, yields a 12-fold activation of the reductase: Phenylmercuric acetate, 6-fold: 8-chloromercuri-2-methoxypropyl urea, 5-fold: and o-[(3-hydroxymercuri-2-methoxypropyl)carbamyl]phenoxyacetic acid, 3-fold. On the other hand, inorganic mercuric ion had no effect on enzyme activity.

Major Findings (Continued):

It would appear that this sulfhydryl group is not directly concerned with the active site of the enzyme since up to 25-fold excess of either cosubstrate, dihydrofolate or NADPH, has no effect on the rate of interaction between the enzyme and organic mercurial.

Merely binding an organic residue to this sulfydryl group is not sufficient in itself to induce this activation of dihydrofolate reductase since the binding of thionitrobenzoate results in inhibition rather than activation. Similarly, other sulfhydryl reagents such as iodoacetate and N-ethylmaleimide do not affect the enzyme.

It is assumed that the binding of certain legends to the SH group induces a conformational change in the protein that enhances its catalytic properties. This probably occurs by affecting a rate limiting step involving either the binding of the substrates or the release of the products of the reaction from the enzyme. However it would appear that such a conformational change is small or localized since examination of the enzyme protein by a variety of techniques such as fluorescence and circular dichroism revealed no significant change in the protein on mercurial activation.

Significance to Bio-Medical Research and the Program of the Institute: Dihydrofolate reductase is not only a key enzyme in the metabolism of folic acid, it is also of major importance in the synthesis of DNA via the biosynthesis of thymidylate. Thus an inhibition of dihydrofolate reductase causes a depletion of tetrahydrofolate leading in turn to a deficiency in thymidylate. Such a deficiency can lead to a cessation of growth in rapidly proliferating cells such as tumor cells. Since a variety of dihydrofolate reductase inhibitors are clinically useful as antibacterials, anti protozoals, immunosuppressants and antineoplastic agents, and understanding of the structure and function of this enzyme would be of basic importance in both normal metabolism and clinical medicine. As yet, many of these basic questions remain to be answered.

Proposed Course of Project: Now that the basic properties of the homogeneous protein are known for both the beef and chicken dihydrofolate reductases, studies will continue to be directed toward the investigation of the interaction of the componants of the reaction with the enzyme protein. In addition, studies will continue as to the role of the various functional groups of the protein in the binding and enzymatic activity. In collaboration with several other groups of investigators, attempts will be made to determine the amino acid sequence and crystal structure of these animal dihydrofolate reductases.

Publications:

Kaufman, B.T. and Kemerer, V.F.; Characterization of Chicken Liver Dihydrofolate Reductase after Purification by Affinity Chromatography and Isoelectric Focusing. Arch. Biochem. Biophys. 179, 420, 1977.

| SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space) | U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT** | PROJECT NUMBER Z01-AM 15300-12 LNE |
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PERIOD COVERED
July 1, 1976 through September 30, 1977.

TITLE OF PROJECT (80 characters or less)

Biochemical Studies Related to Nutrition

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: J. G. Bieri, Chief, Nutritional Biochemistry Sec., LNE, NIAMDD

Other: G. L. Catignani, Staff Fellow, Nutritional Biochemistry Sec., LNE,NIAMDD
T. J. Tolliver, Chemist, Nutritional Biochemistry Sec., LNE, NIAMDD

COOPERATING UNITS (if any)
None

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Nutritional Biochemistry Section

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The liver binding protein for α-tocopherol (vitamin E) was found to have high specificity for d-α-tocopherol when compared with several analogs. Hydrolysis studies showed that the protein is not a lipoprotein or nucleoprotein. Its presence in liver of six species of laboratory animals was demonstrated, but in no other tissue. Tocopherol bound to liver protein was no more effective as an antioxidant than free tocopherol. The nutritional selenium status of rat did not alter the amount or binding capacity of the protein.

Investigation of factors that affect the exchange of α-tocopherol between red blood cells and plasma in vitro showed that the proportion of cells to plasma (hematocrit) and the concentration of total lipids in plasma both had marked effects. At low hematocrits, the concentration of tocopherol in red cells was increased while with high plasma lipids (constant hematocrit) the concentration of tocopherol in red cells was greatly reduced.

Project Description:

Objectives - To determine the nutritional, biochemical and physiological role of essential nutrients for experimental animals. To define the metabolic function of certain nutrients and to study nutritional effects on the tissue levels of various metabolites.

Methods Employed - Rats, hamsters and guinea pigs are fed specially prepared, highly purified diets that contain adequate amounts of each nutrient known to be required by the particular species. The effects of specific deficiencies and imbalances are assessed by measurement of physiological, chemical and enzymological changes in the animal, its tissues and excreta.

Major Findings - Further studies characterizing the α-tocopherol binding protein (TBP) in rat liver supernatant have been carried out. Last year's report presented data characterizing the protein nature of the binding component, its molecular weight and optimal assay conditions, and its high affinity and specificity for α-tocopherol. The chemical nature of the interaction of tocopherol with the binding protein has been further assured by treatment of liver supernatant with various hydrolytic enzymes and assaying for TBP. Pronase completely destroyed binding while trypsin had very little effect. Treatment with RNase, DNase, triacylglycerol lipase C had no effect on binding indicating that TBP is either not a nucleoprotein or lipoprotein or that such components are not necessary for binding.

Competition experiments varying the molar excess of d- or dl-α-tocopherol and several other tocopherols or derivatives demonstrated that the binding protein exhibits stereospecificity for d-α-tocopherol, the naturally occurring isomer. Extraction of radioactivity from both labeled supernatant and partially purified TBP (Sephadex G100 peak fractions) followed by thin layer chromatography, demonstrated that the binding protein carries labeled α-tocopherol as the bound ligand. Preliminary experiments indicate that following an *in vivo* dosing of vitamin E deficient rats with $^{3}$[H]-α-tocopherol and subsequent isolation of liver supernatant, the tocopherol is bound to TBP *in vivo*. Possible transfer of α-tocopherol among subcellular compartments during homogenization was eliminated as the source of tocopherol for binding by incubation of samples at both 0°C and 26°C. Since the rate of association is temperature dependent, introduction of tocopherol by homogenization would result in greater binding at 26°C. Approximately 50% more binding took place, however, at 0°, suggesting that tocopherol was bound *in vivo* and that the rate of dissociation had been slowed by the lower temperature.

TBP was found in male and female liver but not in testis or uterus. It was not detectable in heart, lung, kidney, intestinal mucosa, muscle, brain bone marrow, serum or erythrocyte hemolysate. The binding protein is present in the liver of several laboratory animals including mouse, hamster, guinea pig, rabbit, chicken and monkey. TBP is also present in fetal liver but not in fetal lung or kidney. In other studies the ability of α-tocopherol to prevent lipid peroxidation of liver microsomes was not enhanced by its binding to TBP. The specific activity of TBP whether expressed as tocopherol bound

per gram of tissue or per milligram of protein was unaffected by the selenium nutritional status of the rat.

In other studies of vitamin E metabolism, factors which influence the distribution of α-tocopherol between red blood cells and plasma were investigated. Red blood cells from vitamin E deficient rats were incubated for 6 hours in rat plasma containing $^{14}$C-α-tocopherol, and the distribution of the radioactivity in the two compartments determined (RBC:plasma ratio). It was found that in a model system using bovine serum albumin, about ten times more tocopherol was taken up by red cells than when rat plasma was used. Using rat plasma, the use of heparin as anticoagulant caused a five-fold increase compared to using citrate-dextrose as anticoagulant.

When the hematocrit of the incubations was varied, the RBC:plasma ratio was inversely related to the hematocrit. Of particular significance was the finding that the magnitude of plasma total lipids affected the distribution of tocopherol between cells and plasma. At elevated plasma lipic concentrations the RBC:plasma ratio decreased markedly. We interpret this as a competition between the lipid pools in the two compartments for the fat-soluble vitamin. These studies have significance in the interpretation of blood tocopherol levels as an estimate of vitamin E nutritional status.

Publications:

Bieri, J. G. & Ahmad, K.: Selenium Content of Bangladeshi Rice by Chemical and Biological Assay. J. Agri. & Food Chem. 24: 1073-1074, 1976.

Bieri, J. G. & Farrell, P. M.: Vitamin E. In Munson, P. L., Diczfalusy, E. Glover, J. & Olson, R. E. (Eds.): Vitamins and Hormones. New York, Academic Press, 1976, pp. 31-75.

Bieri, J. G., Evarts, R. P., & Thorp, S.: Factors Affecting the Exchange of Tocopherol Between Red Blood Cells and Plasma. Am. J. Clin. Nutr. 30: 686-690, 1977.

Bieri, J. G., Stoewsand, G. S., Briggs, G. M., Phillips, R. W., Woodard, J. C. & Knapka, J. J.: Report of the Ad Hoc Committee on Standards for Nutritional Studies. J. Nutr. 107: 1977. (in press)

Bieri, J. G.: The Potassium Requirement of the Growing Rat. J. Nutr. 107: 1977. (in press)

Catignani, G. L. & Bieri, J. G.: Rat Liver α-Tocopherol Binding Protein. Biochim. Biophys. Acta 497: 349-357, 1977.

Catignani, G. L.: Role of Vitamin E in Nucleic Acid and Protein Metabolism. In Machlin, L. J. (Ed.): Vitamin E. New York, Marcel Dekker, Inc. (in press)

| SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space) | U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT | PROJECT NUMBER Z 01 AM 15302-07 LNE |
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PERIOD COVERED
July 1, 1976 through September 30, 1977

TITLE OF PROJECT (80 characters or less)
Biochemical Studies of Hepatic and Intestinal Function

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

| | | | | |
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| PI: | Dr. H. G. Windmueller | Research Chemist | LNE | NIAMDD |
| OTHER: | Dr. Ai-Lien Wu | Staff Fellow | LNE | NIAMDD |
| | Mr. A. E. Spaeth | Chemist | LNE | NIAMDD |

COOPERATING UNITS (if any)
None

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|---|---|---|
| 2.3 | 1.3 | 1 |

SUMMARY OF WORK (200 words or less - underline keywords)

The metabolic activity of the small intestine in vivo is being studied in an auto-perfused preparation of rat jejunum in situ developed recently in this laboratory. The rates of uptake and the metabolic fate of nutrients taken up by intestine from the blood or absorbed from the lumen are being quantitatively determined by enzymatic, chromatographic, and radiochemical analyses. Among recent findings are the following: The major circulating substrates and their contribution to respired $CO_2$ in the postabsorptive jejunum were identified as glutamine (35%), 3-hydroxybutyrate (26%), acetoacetate (24%), glucose (7%), lactate (5%), and unesterified fatty acids (3%). All the major carbon and nitrogen end-products of the metabolism of the circulating substrates were identified and quantitated including ammonia, alanine, citrulline, proline, lactate, citrate, $CO_2$, and glucose. Thus glucose is both synthesized and degraded in the gut. Blood and intestinal cell lactate are in very rapid equilibrium, explaining why blood lactate was found to be a quantitatively greater source of carbon for released alanine than endogenously produced lactate. The results provide the first overall view of the intermediary metabolism of the small intestine under physiological conditions in vivo.

Objectives: To increase our understanding of the biochemical transformations occurring in the liver and small intestine. To define the nutritional requirements of these organs, to study the effects of hormones and nutritional variables, and to study the physiological relationship of these organs to other tissues in the body.

Methods employed: Surgical and biochemical techniques are used. Biochemical determinations are made on rat livers and preparations of small intestine vascularly perfused with blood under physiological conditions. Also used are rats surgically prepared with indwelling lymph or bile cannulae as well as a supra-diaphragmetic rat preparation. The metabolic and transport activity of the organs is assessed by the use of radioactive isotopes and measurements of tissue and blood enzyme and metabolite concentrations. Tissue enzymes are localized and characterized.

Major Findings: Studies are continuing to elucidate the major metabolic pathways of the small intestine *in vivo*, by identifying and quantitating the uptake rate and metabolic fate of the major metabolic substrates utilized by intestine from both the blood and from the gut lumen. These studies are made possible by a unique auto-perfused preparation of rat jejunum *in situ* developed recently in this laboratory. Previous studies with this preparation revealed that large quantities of plasma glutamine are metabolized by intestine in addition to glutamate, aspartate, arginine and glutamine absorbed from the lumen. The metabolic end-products resulting from the degradation of these amino acids were identified and their quantitative significance determined.

In a fasting animal, however, the only source of nutrients for the intestine is the blood. Surprisingly, the major circulating substrates for intestinal energy production have not been identified. It has long been known that glycolysis is a very active process in intestinal preparations incubated *in vitro*, and glucose has therefore been generally assumed to be the major energy producing substrate *in vivo*. Studies with our jejunal segment preparation have now clearly shown that this is not the case. Two kinds of experiments were done. (1) Arteriovenous difference measurements were made across rat jejunum to quantitate the net uptake and release of the major known circulating substrates ($O_2$, $CO_2$, glucose ketone bodies, fatty acids, organic acids, ammonia, and various amino acids). (2) When net uptake of a substrate from blood was observed, its metabolic fate in the intestine was quantitatively determined by continuously infusing the compound, labeled with $^{14}C$, into the arterial blood supply to a jejunal segment while measuring the steady-state rate of appearance of $C^{14}$-labeled products in the complete collection of venous blood. To our knowledge, such a comprehensive analysis has not been previously reported for any animal tissue *in vivo*.

The following are among the findings: (1) Approximately 50% of the $CO_2$ produced by the postabsorptive intestine is derived from circulating ketone bodies, identifying them as major oxidative substrates for the gut. Acetoacetate and 3-hydroxybutrate contribute about equally. Another 35% of the $CO_2$ is derived from circulating glutamine. The remaining 15% is derived from glucose, unesterified fatty acids, and lactate. (2) 85% of the amide nitrogen of glutamine is released into blood as ammonia and 75% of the amino

nitrogen as alanine. The remaining nitrogen appears in citrulline and proline, newly-identified as additional end-products of intestinal nitrogen metabolism. (3) Intestine normally exhibits a net release of lactate, but a net uptake is observed when circulating lactate concentrations are abnormally elevated, as in the metabolic lactacidosis accompanying ether anesthesia. Circulating lactate provides most of the carbon for alanine released by the gut. Intestinal cells appear to be extremely permeable to lactate, so that the cellular and blood lactate pools are in virtually instant equilibrium. (In separate experiments it was shown that there is also complete mixing of the pools of lactate and 3-hydroxybutyrate in plasma and blood cells within 30 seconds, the earliest time that measurements were possible). (4) Glucose and glutamine are utilized in about equimolar quantities, but 55% of the glutamine is oxidized to $CO_2$ and only 11% of the glucose, which is largely metabolized to lactate and alanine. The energy contribution from glucose is therefore minor. Furthermore, intestine can synthesize glucose in small amounts, the carbons being contributed by all substrates that feed into the tricarboxylic acid cycle (e.g. aspartate, glutamine, glutamate, lactate, and ketone bodies). (5) The amounts of glucose, glutamine, and ketone bodies taken up and lactate released, based on the arteriovenous difference measurements, were completely accounted for by the rates of metabolic conversion determined in the experiments with $^{14}C$-labeled metabolites. Product analysis reveals some of the salient features of intermediary metabolism in jejunum: an active glutaminase (confirmed by enzyme analysis), glutamate-pyruvate transaminase, and tricarboxylic acid cycle; the presence of the complete pathway for gluconeogenesis; a relatively weak or inhibited pyruvate dehydrogenase; and an incomplete urea cycle, with citrulline as end-product.

Significance to biomedical research and the program of the Institute: These studies enhance our understanding of the digestive process and the intermediary metabolism of the small intestine under physiological conditions in the intact organ. They provide the methodology and basic information needed for subsequent analyses of intestinal function under altered physiological or pathological conditions.

Proposed Course: This unique intestinal preparation will be used to further elucidate metabolic events during nutrient absorption. When used in conjunction with intestinal lymph collection, the preparation is also providing new information regarding the intestinal biosynthesis of the protein moieties of chylomicrons and other circulating lipoproteins.

Publications:

Windmueller, H. G. and Spaeth, A. E.: Metabolism of Absorbed Aspartate, Asparagine and Arginine by Rat Small Intestine in vivo. Arch. Biochem. Biophys. 175: 670-676, 1976.

Windmueller, H. G. and Spaeth, A. E.: Vascular perfusion of rat small intestine: metabolic studies with isolated and in situ preparations. Fed. Proc. 36: 177-181, 1977.

Pinkus, L. M. and Windmueller, H. G.: Phosphate-dependent glutaminase of small intestine: Localization and role in intestinal glutamine metabolism. Arch. Biochem. Biophys. (in press)

| SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space) | U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT** | PROJECT NUMBER Z01-AM 15400-03-LNE |
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PERIOD COVERED
July 1, 1976 to September 30, 1977

TITLE OF PROJECT (80 characters or less)
Hormones, lipoprotein lipase and fat metabolism

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

| | | |
|---|---|---|
| PI: | Peter M. Spooner, Staff Fellow | LNE NIAMDD |
| OTHER: | Robert O. Scow, Chief, Section on Endocrinology | LNE NIAMDD |
| | Sidney S. Chernick, Scientist Director | LNE NIAMDD |

COOPERATING UNITS (if any)

LAB/BRANCH
Laboratory of Nutrition and Endocrinology

SECTION
Endocrinology

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20014

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

A primary cell culture has been developed from rat mammary glands which is suitable for the study of hormonal regulation of lipoprotein lipase activity. Enzyme digests of mammary glands from rats in midpregnancy are separated into five density fractions on Ficoll gradients. The cells of each fraction are maintained in a complex hormone-supplemented medium. Lipoprotein lipase activity of each of the cell fractions is initially low but that of Fractions I and II, which accumulate large lipid droplets, increases 10-20 fold between the nineth and twelfth day of culture. Nutritionally selected cells of Fractions I and II produce lipoprotein lipase in the presence of prolactin plus a limited combination of hormones.

These preliminary findings suggest that in culture a specific epithelial cell type in mammary gland produces lipoprotein lipase in response to hormones.



PHS-6040
(Rev. 10-76)

Project Description:

Objectives: To determine how hormones regulate lipoprotein lipase activity in cells of mammary gland. To develop a culture system for mammary gland cells that supports differentiation of function in response to hormones in vitro.

Methods Employed: Enzyme digestion of mammary glands from rats in mid-pregnancy is used to obtain free cells. The cells are separated in five different density fractions on linear Ficoll (Pharmacia) gradients. Cells of each fraction are grown to confluence in a complex hormone-supplemented medium. At intervals of 3-5 days cells are assayed for lipoprotein lipase activity by methods developed in this laboratory. Protein and other determinations are made as required. The lipase has been identified by enzymatic and immunological procedures. Fractions I and II (low density fractions) increase 10-20 fold between 9 and 12 days of culture. These fractions have epithelial morphology and accumulate large lipid droplets. Fraction III appears to be fibroblasts. Fractions IV and V are derived from ductal and alveolar secretory elements and have very little lipoprotein lipase activity.

Major Findings: Mechanism of hormonal regulation of lipoprotein lipase activity in mammary gland. Lipoprotein lipase activity is increased in mammary gland and decreased in adipose tissue during lactation. Earlier studies showed that these changes in enzyme activity are mediated through prolactin secretion by the anterior pituitary gland. The present study was initiated to determine the mechanism of hormonal regulation of lipoprotein lipase activity in mammary gland cells.

During the past year we have developed a suitable primary mammary cell culture system with which hormonal regulation of lipoprotein lipase activity can be studied. Initial experiments showed that lipoprotein lipase activity is initially low in all cell fractions but undergoes a selective 10-20 fold increase in Fraction I and II cells between the 9th and 12th days of culture. These cells, which are found in the lightest fraction of the gradient (d = 1.007-1.070), also accumulate large intracellular lipid droplets and have an epithelial morphology. They can be propogated in a growth medium which selects against fibroblasts and appear distinct from mammary fibroblasts, which are found in Fraction III of the gradient. The lipase in Fraction I and II cells has been identified as lipoprotein lipase by using a specific antibody to rat heart lipoprotein lipase. Thus far, very little lipase activity has been found in the Fraction IV and V cells which appear to be derived from ductal and alveolar secretory elements.

Recent experiments using nutritionally selected Fraction I and II cells have shown that lipoprotein lipase activity is inducible with prolactin plus a limited combination of other hormones. Present studies are directed at determining the specific sequence of hormonal events required for the response and confirming the physiological identity of the cells involved.

Preliminary findings suggest that there may be a specific cell type in mammary gland which produces lipoprotein lipase in response to hormonal signals prior to parturition and throughout lactation.

Significance to Biomedical Research and the Program of the Institute. Lipoprotein lipase is necessary for uptake of triacylglycerol from blood by extrahepatic tissues. Earlier studies in this laboratory showed that the enzyme acts in capillary endothelium to hydrolyze triacylglycerol to partial glycerides and fatty acids which cross the capillary to be utilized by tissue cells. Uptake of chylomicron cholesterol by extrahepatic tissues is also dependent on lipoprotein lipase activity. We have proposed a model for transport of partial glycerides, fatty acids and cholesterol from chylomicrons to cells in tissue by lateral diffusion in a continuous water-lipid interfacial plane consisting of the chylomicron surface film and the external leaflets of plasma and intracellular membranes of endothelial, interstitial and parenchymal cells. It was also proposed that lipids derived from chylomicrons leave the interfacial continuum in parenchymal cells where they are esterified, to either triacylglycerol or cholesterol ester, and separate from the lipid interface to accumulate between the external and cytoplasmic leaflets of endoplasmic reticulum. It is likely that this mechanism for transport and accumulation of lipid in extrahepatic tissues operates in the walls of large blood vessels (see also project Z01-AM 15401-05-LNE).

The present study contributes to this thesis information on the site of synthesis of lipoprotein lipase and may assist in clarifying the paradox that the enzyme, which presumably acts only at the luminal surface of capillaries, may be synthesized solely in the parenchymal cells far remote from its site of action.

Proposed Course. The mechanism of action of prolactin in the regulation of lipoprotein lipase activity will be studied in rodent mammary tissue in tissue culture. The formation of immuno-identifiable enzyme will be related to lipase activity. The location of enzyme in tissues and cell cultures will be made by use of immuno-fluorescent histological procedures. The mechanism of suppression of lipoprotein lipase activity in adipose tissue of lactating rats and in mammary glands of non-suckling rats will be explored in vivo and in vitro.

Publications:

Spooner, P.M., Garrison, M.M. and Scow, R.O.: Regulation of mammary and adipose tissue lipoprotein lipase and blood triacylglycerol in rats during late pregnancy: Effect of prostaglandins. J. Clin. Invest. In press.

| SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space) | U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT** | PROJECT NUMBER Z01-AM 15401-05-LNE |
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PERIOD COVERED
July 1, 1976 to September 30, 1977

TITLE OF PROJECT (80 characters or less)

Transport of lipids, hormones and enzymes in tissues, cells and membranes

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT


PI: Robert O. Scow, Chief, Section on Endocrinology LNE NIAMDD
E. Joan Blanchette-Mackie, Research Biologist LNE NIAMDD


COOPERATING UNITS (if any)

Dr. Robert Verger, CNRS, Marseille, France

LAB/BRANCH
Laboratory of Nutrition and Endocrinology

SECTION
Endocrinology

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20014

| TOTAL MANYEARS: | PROFESSIONAL: | OTHER: |
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| 3 | 2 | 1 |

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)


The membranes of lipid droplets in cells are lamellar structures, containing amphilic lipids (fatty acids and partial glycerides), and are continuous with membrane leaflets of smooth endoplasmic reticulum and Golgi. In mammary glands, a mechanism of milk lipid droplet formation is proposed based on electron microscope and freeze-fracture studies. In developing fat cells membrane leaflets are sites for esterification of fatty acids and storage of triacylglycerol. In hepatic cells lamellae of lipid droplets are sites of intracellular lipolysis.

Electron microscope autoradiography demonstrated the route of chylomicron lipids across the capillary wall to cells of lactating mammary gland.

With monolayer technics we studied the action of purified lipoprotein lipase on trioleoylglycerol. Products of hydrolysis moved laterally in a lipid-water interface and both movement and lipolysis are enhanced when the products are removed from the interface at a distal point. The findings support the model proposed for transport of lipids by lateral diffusion in cell membranes.




PHS-6040
(Rev. 10-76)

Project Description:

Objectives: To study the structure and metabolism of cells that synthesize and secrete lipids and hormones. To study the transport of lipids, hormones and enzymes across the capillary endothelium, basememt membrane and extracellular space in different kinds of tissues. To study the uptake of lipids, hormones and enzymes by different kinds of cells. To study the structure of cellular membranes and their role in lipid metabolism. To correlate structural with biochemical changes induced experimentally in cells and tissues. To study the effect of lipoprotein lipase on trioleoylglycerol monolayers and measure lateral diffusion of lipolytic products.

Methods Employed: Animals of different species and different ages and in various physiological states (e.g. fasting, pregnant, lactating) are used to study with biochemical and morphological techniques the synthesis and secretion of lipoprotein triacylglycerol by liver and intestines, and utilization of plasma triacylglycerol by adipose tissue, mammary gland, and muscle. Perfused adipose and mammary tissues are used to locate with cytochemical techniques the sites of action of lipoprotein lipase, the mode of transport of glycerides, fatty acids and cholesterol between blood and tissue cells. Morphology of cellular membranes was investigated with the electron microscope and the freeze-fracture technic. Autoradiological studies of the uptake of labeled chylomicron lipids were performed in lactating rats.

The monolayer technics of Dr. Robert Verger were used to study the action of lipoprotein lipase on triacylglycerol and the movement of lipolytic products by lateral diffusion in a lipid-water interface.

Major Findings: A. Intracellular membranes and lipid. I.Formation, transport and secretion of milk lipid. Electron microscope studies in our laboratory have shown that lipid droplets are intimately associated with intracellular membranes in most cells. These cellular lipid droplets have complex structures composed of homogeneously electron opaque (osmiophilic material) situated within an irregularly arranged lamellar matrix. In vitro studies on chylomicrons determined that the structure visualized with our preparatory procedures reflect the class of lipid molecules within the chylomicron sphere. Amphiphilic molecules (fatty acids and partial glycerides) formed lamellae at the interface between triacylglycerol and water. These amphiphilic lipids are visualized with the electron microscope as linear densities surrounding homogeneously electron-opaque triacylglycerol.

The structure of lipid droplets seen in thin sections of mammary gland secretory cells similarly reflects the physical properties of the component lipid molecules. The homogeneously electron-opaque areas are triacylglycerol and the lamellar matrix is composed of amphiphilic lipids at the interface between triacylglycerol and the aqueous environment. Further, at the periphery of the lipid droplets, lamellae appear continuous with membrane leaflets of smooth endoplasmic reticulum and Golgi. Thus most of the

lamellae within the lipid droplet are thought to be inward extensions of leaflets of membrane bilayers containing triacylglycerol within their hydrophobic hydrocarbon interior. In the lactating mammary gland, the triacylglycerol within leaflets of intracellular membranes is released subsequently to the alveolar lumen as milk lipid. Our structural studies show that in the apical region of the secretory cell, Golgi membrane leaflets enveloping triacylglycerol fuse with the apical plasma membranes releasing the milk lipid package (triacylglycerol and amphiphilic lipid components of the lamellae) to the alveolar lumen.

The significance of these structural findings are: (1) the presence of an interfacial continuum within lipid droplets provides a hydrophobic environment through which presumably liquid triacylglycerol molecules are free to move. Additionally, intracellular translocation of triacylglycerol could be accomplished by an unraveling of lamellae from lipid droplets and their redistribution to other sites within the cell. (2) The interfacial continuum provides also a path for the movement of amphiphilic lipid molecules throughout the cell to sites of esterification. (3) Secretion of milk lipid via the Golgi leaves the apical surface of the alveolar cell covered by Golgi membranes containing specific Golgi enzymes. Such a mechanism dictates that there is, for a finite time a heterogenity of enzyme molecules on the cell surface and suggests that molecular redistribution must subsequently occur. (4) The "membrane" fraction of milk contains lamellae which arise from multiple membrane sites within the cell, are heterogeneous in lipid composition and are secreted as a package with the milk triacylglycerol.

Another methodological approach to visualization of lipids is freeze-fracture. Frozen tissue fractures along hydrophobic planes. Therefore structure at sites where membrane leaflets enclose triacylglycerol could be investigated, as well as structure of the cell surface at the sites of membrane fusion. With this technique we avoided chemical fixation of lipids by quick freezing mammary gland. Our initial results show previously unresolved structure within intracellular lipid droplets. There are aggregation of particles on lipid fracture faces suggestive of intracellular particles within membrane leaflets. These particles may represent specific enzyme molecules within monolayers of the lipid droplet. We are investigating also the sequence of reorganization of membrane components during membrane fusion at sites of milk lipid secretion.

II. Differentiation of adipose cells. Differentiating white adipose tissue from epididymal fat pads of 4 to 6 day old rats was examined with the electron microscope. The presumptive fat pad contained cells of varying morphology, from spindle-shaped fibroblasts with little lipid to mature unilocular cells. In these cells there is abundant endoplasmic reticulum. Lipid droplets of all sizes contain lamellae and amorphous triacylglycerol. Associations between endoplasmic reticulum leaflets and lipid droplets suggest that lamellae of the lipid droplets are continuous with membrane

leaflets. Thus intracellular membranes are the site of both esterification of fatty acid and storage of triacylglycerol within developing adipocytes.

III. Lipolytic activity in liver. In hepatic cells of fed rats, fixed briefly and incubated at 37°C, lipid inclusions showed electron-lucent areas which suggest that enzymatic hydrolysis of triacylglycerol occurs within the droplets. The lamellae present within the droplet may provide the interface for this enzymatic activity.

B. Transport of lipid ($^{3}$H-oleate and $^{3}$H-glyceride triacylglycerol) from capillaries to parenchymal cells: Electron microscopic autoradiography. Previous studies showed that chylomicrons within the capillary bed of lactating rat mammary gland are enveloped by finger-like projections of the capillary endothelium during uptake of triacylglycerol by the tissue. Histochemical studies were interpreted to show that lipolysis of chylomicron-triacylglycerol by lipoprotein lipase occurs at the lumenal surface of the endothelial cell with the release of amphiphilic products (fatty acids and partial glycerides). Electron microscopic autoradiography was used to localize labeled lipid in the tissue, in an attempt to determine the route of transport of chylomicron-fatty acid from the blood to secretory alveolar cells.

Chylomicrons labeled with either glycerol tri[$^{3}$H]oleate glycerol or $^{3}$H-glycerol trioleate were injected into rats lactating 4 days. After 3 minutes mammary tissue was excised and fixed in both osmium tetroxide and/or aldehyde. Some of the aldehyde fixed tissue was further incubated at 37°C in buffer.

Reports in the literature indicate that lipid movement can occur in cells and membranes during and after aldehyde "fixation". This study attempted also to determine if a temporal translocation of lipid in cells of the lactating rat mammary gland occurs after fixation with aldehyde.

The results showed that 3 minutes after injection of chylomicrons into the lactating rat, most of the labeled lipid visualized in the mammary gland, was associated with chylomicrons in the capillary lumen, and also with intracellular and intralumenal milk lipid droplets. Some intracellular movement of label (and thus lipid) occurred during aldehyde fixation.

C. Lipid transport by lateral diffusion using monolayer technics. The equivalent of 20 layers of tri-[$^{14}$C]oleoylglycerol was spread over 0.1 M Tris·HCl solution, pH 7.4, at 28-30°C under argon. The surface pressure of the interface increased from 15.8 to 18.0 dyne/cm when a small amount (160 µg) of albumin was added to the subphase, and increased further to 20.0 dyne/cm when 10 µg of C-peptides from HDL were added. Addition of 4.4 µg of purified lipoprotein lipase to the subphase caused an immediate and sustained increase in surface pressure to 29 dyne/cm. Since this value is 5-6 dyne/cm less than the collapse pressure for a monolayer of either dioleoylglycerol, monoleoylglycerol or oleic acid, the increase in pressure

indicates that trioleoylglycerol was hydrolyzed by lipoprotein lipase and that products of lipolysis displaced trioleoylglycerol and, probably, the protein components from the interface. However, when a large amount (64 mg) of albumin was added to the subphase 2 min after adding lipoprotein lipase, the surface pressure increased only to 24 and then fell to 23 dynes/cm, suggesting that lipolytic products (probably oleic acid and monoleoylglycerol) were being removed from the interface by the large amount of albumin. (Preliminary studies showed that albumin in the subphase could remove oleic acid and, to a lesser extent, monoleoylglycerol but not diacylglycerol from the interface between argon and Tris.HCl solution, pH 7.4).

Transport of lipolytic products by lateral movement in the triacylglycerol-water interface was then studied with a trough having two compartments connected by a narrow surface canal. The volume and exposed surface of compartment A were each 3 times that of compartment B. The equivalent of 20 layers of tri-[$^{14}$C]oleoylglycerol was spread over both compartments; 160 µg of albumin, 10 µg of C-peptide and 4.4 µg of lipoprotein lipase were added to compartment A; and 64 mg of albumin, when used, was added only to compartment B. Aliquots were taken at various intervals from the subphase of each compartment, and analyzed for lipid composition. Hydrolysis of trioleoylglycerol by lipoprotein lipase in compartment A was increased several fold, and movement of oleic acid and monoleoylglycerol to compartment B were increased 5-10 fold when albumin had been added to compartment B. The proportion of lipolytic products recovered in compartment B was also increased, from 16 to 33% of the total, when albumin had been added. When an excess of enzyme was added to A and albumin to B, lipolysis was increased slightly but less than 5% of the products were recovered in compartment B. The latter indicates that very little if any enzyme was present in compartment B, and that the interface above B probably consisted of mostly dioleoylglycerol, which would not be removed by albumin. (Analyses of the interface above each compartment are being done.)

These studies show that (1) products of hydrolysis of triacylglycerol can locate and move laterally in a lipid-water interface, and that (2) lipolysis and (3) movement in the interface are both enhanced when products of lipolysis are removed from the interface at some point distal to the enzyme. These findings support the model proposed above for the transport of lipid by lateral diffusion in cell membranes.

Significance to Biomedical Research and the Program of the Institute. Lipoprotein lipase is necessary for the uptake of chylomicron triacylglycerol from blood by extrahepatic tissues. Other studies in this laboratory showed that the enzyme acts in capillary endothelium to hydrolyze triacylglycerol to partial glycerides and fatty acids which cross the capillary to be utilized by tissue cells. Recent studies showed that uptake of chylomicron cholesterol by extrahepatic tissues is also dependent on lipoprotein lipase activity. We have proposed a model, based in part on the above findings, for transport of partial glycerides, fatty acids and cholesterol from

chylomicrons to cells in tissue by lateral diffusion in a continuous water-lipid interfacial plane consisting of the chylomicron surface film and the external leaflets of plasma and intracellular membranes of endothelial, interstitial and parenchymal cells. (See Project Z01-15400-02-LNE). It is also proposed that lipids derived from chylomicrons and other sources leave the interfacial continuum in parenchymal cells where they are esterified, to either triacylglycerol or cholesterol ester, and separate from the lipid interface to accumulate between the external and cytoplasmic leaflets of endoplasmic reticuluum. It is likely that this mechanism for transport of lipid in extrahepatic tissues operates in the wall of the large blood vessels. We think that FFA mobilized from adipose tissue as well as amphiphilic proteins and hormones may also move between blood and parenchymal cells by lateral diffusion in the above interfacial continuum. These possibilities are under study.

Proposed Course. We plan to continue use of the freeze etching technique, combined with electron microscopy, to study the transport of lipid from blood to tissue cells, the secretion of lipid by cells (intestinal, liver, mammary gland), and the interaction of lipoprotein lipase with chylomicrons. Immuno-cytochemical techniques will be developed for locating lipoprotein lipase in parenchymal cells and capillary endothelium and on chylomicrons incubated with the enzyme. Monolayer technics learned in Marseille will be set up in our laboratories to continue these studies on lipase action.

Publications:

Mendelson, C.R., Zinder, O., Blanchette-Mackie, E.J., Chernick, S.S. and Scow, R.O.: Lipoprotein lipase and lipid metabolism in mammary gland. J. Dairy Science 60: 666-676, 1977

Scow, R.O., Chernick, S.S. and Fleck, T.R.: Lipoprotein lipase and uptake of triacylglycerol, cholesterol and phosphatidylcholine from chylomicrons by mammary and adipose tissue of lactating rats in vivo. Biochim. Biophys. Acta 487: 297-306, 1977

Scow, R.O., Blanchette-Mackie, E.J., and Smith, L.C.: Role of capillary endothelium in the clearance of chylomicrons: A model for lipid transport from blood by lateral diffusion in cell membranes. Circ. Res. 39: 149-162,1976.

Scow, R.O. and Egelrud, T.: Hydrolysis of chylomicron phosphatidylcholine in vitro by lipoprotein lipase, phospholipase $A_2$ and phospholipase C. Biochim. Biophys. Acta 431: 538-549, 1976.

Zinder, O., Mendelson, C.R., Blanchette-Mackie, E.J. and Scow, R.O. Lipoprotein lipase and uptake of chylomicron triglyceride and cholesterol by perfused mammary tissue. Biochim. Biophys. Acta 431: 527-537, 1976.

Scow, R.O., Blanchette-Mackie, E.J. and Smith, L.C.: Role of lipoprotein lipase and capillary endothelium in the clearance of chylomicrons from blood: A model for lipid transport from blood by lateral diffusion in cell membranes. Exposes annuels de biochimie. medicale 33: 143-164, 1977.

| SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space) | U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT** | PROJECT NUMBER Z01-AM 15402-03-LNE |
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PERIOD COVERED
July 1, 1976 to September 30, 1977

TITLE OF PROJECT (80 characters or less)
Pharyngeal lipase and digestion of dietary fat

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: Robert O. Scow, Chief, Section on Endocrinology LNE NIAMDD
Sidney S. Chernick, Scientist Director LNE NIAMDD
Other: Peter M. Spooner, Staff Fellow LNE NIAMDD

COOPERATING UNITS (if any)

LAB/BRANCH
Laboratory of Nutrition and Endocrinology

SECTION
Endocrinology

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20014

| TOTAL MANYEARS: | PROFESSIONAL: | OTHER: |
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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The purpose of this project is to characterize the lipase that initiates the hydrolysis of dietary fat. This "pharyngeal lipase" produces amphiphilic lipids in the stomach as the first step in the digestion of fat. The enzyme has been partially purified from serous glands of the tongue in rat and from the secretions of a man with esophageal fistula. In both the enzyme activity is present in 2 elution peaks by gel chromatography with estimated molecular sizes of $2x10^5$ and $2x10^6$. These may represent polymeric or adsorbed forms of the enzyme. In purified form the enzyme is labile whereas in crude form (juice or frozen glands) it is very stabile.

Further efforts will be directed towards characterizing the enzyme and investigating the physiological regulation of its secretion. This enzyme could play an important role in fat digestion in infants.

Project Description:

Objectives: To purify the pharyngeal lipase in esophageal secretions of man and to compare it to the lipase present in serous glands in the rat tongue.

Methods Employed: Serous glands at the base of the tongue of rats are dissected and homogenized in appropriate buffers (pH 5.4) with the aid of a Polytron (Brinkmann Inst.). Enzyme is present in the high speed supernate and particulate fractions. Soluble enzyme is subjected to gel chromatography on Sephadex G100, G200 or Sepharose 6B with appropriate buffered eluting solutions. Protein and enzyme activity of the various fractions are measured. Purification by isoelectric focusing on carrier ampholytes in a continuous pH gradient was attempted on pooled active fractions.

Major Findings: The assay for pharyngeal lipase was improved by the development of a stock triglyceride-amphiphilic lipid suspension in glycerol. Dilution of the stock suspension with buffered albumin solutions produced uniform emulsions of the labeled triacylglycerol substrate. The stock solution is stable for at least one year.

Liquifying the heavy mucous in esophageal juice by sonication or lyophilizing and extracting the enzyme with 0.1 M citrate buffer, pH 5.4 resulted in a clear high speed supernate with relatively high enzymatic activity. Gel filtration on Sephadex G200 gave two peaks of lipase activity (molecular size $>2x10^5$ and $10^6$). Concentrating the fractions by ultrafiltration (S and S collodion bags or Millipore Immersible Molecular Separator) resulted in 90% loss of activity. The heavy insoluble precipitate that formed suggested that a large amount of mucoprotein was present in these fractions. Isoelectric focusing of the most active peak ($90\text{-}100x10^3$) concentrated the lipase at pH 6.5. However only a small fraction of the activity applied to the column was recovered.

Further efforts were directed towards the enzyme in rat tongue. Gel filtration of active extracts on Sepharose 6B also produced two active peaks with molecular sizes of about $10^6$ and $10^5$ daltons. These purified preparations are much less stable than the crude extracts and lipase activity is lost when samples are lyophilized, concentrated by ultrafiltration or diluted. The ion concentration does not appear to be an important factor, but pH is.

From the kinetics of enzyme action the human and rat enzymes appear to be very similar. Present work involves finding better methods of purifying and storing the enzymes prior to determining their kinetic parameters.

Significance to Biomedical Research and the Program of the Institute: Pharyngeal lipase accounts for the lipolytic activity in gastric contents and the products formed, especially monoacylglycerol and fatty acids, facilitate emulsification of triacylglycerol before it leaves the stomach.

The importance of this enzymic reaction in patients with abnormal fat digestion is not yet known.

Proposed course: Studies will be continued to determine the role of pharyngeal lipase in fat digestion and to determine what factors regulate its synthesis and secretion. Studies will also be made to determine the site of synthesis and secretion of pharyngeal lipase in man. Isolation and purification of the lipase from tissues of rats and from secretions of man will be continued.

Publications: none

| SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space) | U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT** | PROJECT NUMBER Z01 AM 15500-17 LNE |
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PERIOD COVERED

July 1, 1976 through September 31, 1977

TITLE OF PROJECT (80 characters or less)

Large-scale Processing of Biological Materials.

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: Mr. D. L. Rogerson, Jr. Head, Pilot Plant LNE NIAMDD
OTHER: None

COOPERATING UNITS (if any)

None

LAB/BRANCH

Laboratory of Nutrition and Endocrinology

SECTION

Office of Chief

INSTITUTE AND LOCATION

NIAMDD, NIH, Bethesda, Maryland, 20014

| TOTAL MANYEARS: | PROFESSIONAL: | OTHER: |
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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Production of large quantities of microorganisms for the isolation and purification of biologically important macromolecules and cell particulates and to assist NIH investigators in the scale-up and processing of plant and animal tissue for biological substance such as enzymes, vitamins, alkaloids, etc.



PHS-6040
(Rev. 10-76)

Project Description

Objectives: To provide facilities and services for the processing and/or production of large amounts of natural materials, such as bacteria, animal tissues, cells, blood, urine and plant materials, so that they may be studied under standard laboratory conditions.

Methods Employed: To satisfy the large-scale production needs of NIH investigators, methods are designed for the scaling up of small laboratory experiments utilizing large reaction vessels, extractors, stills, concentrators, centrifuges, etc., so that the processing can be carried out effectively, efficiently and safely. Two 10-liter, one 50-liter, two 300-liter and one 1100-liter fermentors are utilized for growing microorganisms under a variety of conditions. Bacterial cells and yeasts are harvested by means of laboratory or large-scale AS-16P and AS-26P centrifuges. Protozoa are concentrated in continuous flow centrifuges. Cells are either supplied as a paste or further processed to yield cell-free suspensions by rupture with a Gualin homogenizer. In some instances, a specific substance is isolated and partially purified by further processing of the culture supernatant or the cell paste using conventional fractionation procedures and the newer chromatographic techniques.

Major Findings: During the past 15 months, a total of 393 requests were processed for investigators: NIAMDD 193; BoB 52; NCI 35; NHLBI 40; NICHD 22; NIDR 17; NIMH 11; NINCDS 8; NIAID 8; and others 7. 152.5 Kg quantities (44,500 liters of cultured of 29 organisms were produced. These organisms grown include wild type and mutant strains of Arthrobacter luteus, Arthrobacter polychromogenes, Aspergillus oryzae, Azotobacter vinelandii, Bacillus amyloliquifaciens, B. globiggin, B. pumilus, B. stearothermophilus, B. Subtilis, Breorbacterium albidum, Candida utilis, Escherichia coli, Euglena graciiis, Haemophilus influenzae, H. parainfluenzae, Namalva Blymphocytes, Neisseria Meningitidis, Pneumococcus sp, Pseudomonas sp, Succharomyces cerevisiae, Salmonella typhimurium, Staphlococcus aureus, Strytococcus mutans, Streptococcus sp., Strephtomyces stanfordi, Tetrahymena pyriformis, Xanthomonas holcicola, Zymobacterium oroticum and PS-3 and unidentified thermophile. Some of the organisms were used for the production of bacteriophages T4, T7, fd, lambda and PM2, others were used to produce extracellular enzymes. Microorganisms were grown 70 times in the two 10-liter, 92 times in the 50-liter, 19 times in the new 100-liter, 84 times in the 300-liter and 11 times in the 1100-liter fermentors. Of the 276 fermentations, 24 (8.7%) were contaminated with 13 (4.7%) of these traced to the inocula supplied by the requesting investigator.

Studies were continued with the Division of Bacterial Products, Bureau of Biologics for polysaccharide production by H. influenzae and isolation of the enterotoxin proteins from special strains of E. coli. Sphecoplast were produced for the NHLBI from E. coli using 10-,50-, and 300 liter fermentors in series. Crude interferon fractions were isolated from 200 liter cultures consisting of transformed β-lymphocytes stimulated with Newcastle disease virus, Strain B-1. An attempt at producing interferon from an 800 liter culture is in progress. The Gaulin laboratory homogenizer was used 34 times to rupture cells suspensions of yeast, protozoa or bacterial cells.

Large scale processing activities consisted of the isolation of histamine methylating enzyme from two 1800 g lots of guinea pig brains for NHLBI; microsomes from twelve lots of chicken livers (10-45 lbs) using the Electro-nucleonics zonal centrifuge (also NHLBI); isolation of an extracellular enzyme, Barnase, from two 1100-liter cultures of B. Amyloliquefaciens; extraction of coagulation factor 13 from 8 Kg placentas; and preparation of seven two-gallon quantities of hydroxylapatite. Various plants and animal tissues were either dried, and/or ground in the large ovens, mills, lyophilizer and related equipment and solutions were centrifuged such as 80 liter of Kilham rat virus cultures, 88 liters of Bordetella pertussis cultures and several 10-15 liter cultures of E. coli mutants and their viruses.

Publications: None

| SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space) | U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT** | PROJECT NUMBER ZO1 AM 15600-22 LNE |
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PERIOD COVERED

July 1, 1976 through September 31, 1977

TITLE OF PROJECT (80 characters or less)

Studies in Experimental Nutrition

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

| | | | |
|---|---|---|---|
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SECTION

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| TOTAL MANYEARS: | PROFESSIONAL: | OTHER: |
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| 1.5 | 1.0 | 0.5 |

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

It has been shown that bone zinc can become available to maintain zinc nutrition during extended periods of zinc-free feeding. Periods of normal growth, freedom from deficiency symptoms and survival time were related to levels of bone zinc which were in turn related to the amount of zinc administered during a zinc loading period in the first ten days after weaning. Bone zinc decreased from relatively high levels during the period of observation and reached deficient levels when s toms became evident. Germ free rats appear to require less zinc than non-germfree rats, female rats apparently require less zinc than males especially in a germfree state. Although the germfree state did not markedly delay onset of vitamin A deficiency, it did result in somewhat less severe symptoms and increased survival time. A germfree state apparently increases food efficiency for rats with limited food intake but not for ad-lib fed rats. By dietary means we have produced rats exhibiting severe symptoms of tryptophan deficiency, including marked hypersensitivity, tremors, seizures and decreased levels of 5-hydroxytryptamine in brain and other organs. Hopefully these will be suitable models to attempt to correlate the abnormal behavior with alterations in the biochemistry of neuronal systems.

Project Description

With Dr. Yamamoto we have continued experiments, started under Dr. Laqueur, relating to the carcinogenic effects of cycasin and cycasin-related compounds, and the influence of bacteria upon the toxicity of these compounds. Cycasin itself produces tumors in conventional rats and in newborn germfree rats but has no effect in older germfree rats. Methyl azoxymethane (MAM), the aglycone of cycasin was found to cause tumors in germfree as well as in non-germfree rats. Experiments with azoxymethane (AOM) and dimethylhydrazine (DMH) have been completed and were observed to cause tumors in bermfree as well as in non-germfree rats, and must themselves be active or be converted to active forms by the animal tissues. The clucuronide of MAM if now being studied but sufficient time has not elapsed to draw meaningful conclusions. It has been observed that the degree of toxicity and the location of tumors formed with the various compounds are not identical, indicating that the various compounds may themselves be toxic or converted to toxic compounds of various compositions, and may not require complete conversion to the aglycone. It is hoped to identify which compounds in the in vivo conversion of cycasin to its aglycone are carcinogenic and which of these may be formed by the animals own enzymes. It is further hoped to identify specific bacteria responsible for those compounds which occur only as the result of bacterial action.

With Dr. Smith experiments wer continued to study the requirements and utilization of the trace mineral zinc, and its relationship to the utilization of vitamin A. Earlier it was shown that in the absence of adequate zinc, although the rat could accept and store in the liver large amounts of Vitamin A, it appeared unable to mobilize and transport sufficient amounts of the vitamin to maintain normal levels in the plasma. It was also shown that this inability to handle vitamin A in a normal manner was due in part to the lack of growth which occurs in the absence of adequate zinc, and that growth inhibition by other means, such as food restriction, also resulted in a similar inability to normally utilize vitamin A even in the presence of adequate zinc. Plasma zinc was also decreased during growth restriction, but this could be overcome by injecting rather large amounts of zinc daily. Maintaining normal plasma zinc levels in this manner did not completely correct the apparent inability to utilize vitamin A.

Groups of animals were fed a complete diet in controlled daily amounts to permit growth rates ranging from zero growth to normal growth with ad-lib feeding. Zinc and vitamin A were administered orally or by injection to insure adequate intake of these two factors. It appeared that any amount of growth restriction below maximum growth decreased the plasma zinc below normal and about equally. Plasma A however was decreased progressively lower as the amount of food permitted was decreased. These two factors did not respond to different levels of food intake and growth in the same amnner.

Conventional rats were fed vitamin A deficient diets containing adequate zinc. For half of the animals food intake was limited to allcw growth to a maximum of about 90 grams. The remaining rats were permitted unrestricted food intake and growth. In approximately six weeks ad lib fed animals had gained about 180 grams, then growth leveled off and dificiency symptoms including crippling and eye lesions occurred in all the animals. Death occurred in these

Project Description (Continued):

rats from about the sixth to the tenth week. Animals which were limited in food intake gained about 65 grams during the same six week period and about 70% of the group showed similar dificiency symptoms but somewhat less marked than for the ad lib group, and survival time was not markedly increased.

Similar experiments were done with germfree and ex-germfree rats. Here as in the above experiments time of crippling was not markedly delayed by limiting growth in either the germfree or ex-germfree groups, but the severity of crippling was somewhat less marked for limited growth groups in both germfree and ex-germfree rats. Survival times were improved for both restricted and ad lib fed rats in the germfree group when compared with non-germfree animals. Since the onset of deficiency symptoms was not markedly delayed by the germfree state, it is suggested that the increased survival times may be due to the absence of stress of infection, rather than to a more efficient utilization or conservation of vitamin A stores.

It was interesting that in the above experiments the food efficiency appeared to be about equal for germfree and ex-germfree rats on the unrestricted diet, but for the restricted group the ex-germfree rats required about 20% more food to produce the same amount of body weight gain as did the germfree rats.

In a number os experiments done by loading rats with large amounts of vitamin A then administering varying doses of zinc daily, the plasma A appears to be maintained in the normal range only when growth was permitted, even though normal plasma zinc levels could be maintained with the higher doses of zinc. This was true whether they were first made doubly deficient or started at weaning.

To determine if bone zinc can become available to the rat as a source of zinc for normal requirements experiments were done in which the bone was loaded with zinc in a relatively short period. Zinc was administered intraperotineally over a 10 day period in amounts ranging from 0 to 1000 micrograms per day. Some animals were sacrificed following the 10 day "build up" period and others were permitted to continue without further zinc for an additional three weeks then sacrificed. It was observed that amounts less than 100 micrograms per day were insufficient to establish a normal plasma zinc level, although bone zinc was elevated gradually with increases in dosage. After the build-up period the zero zinc group continued to become even more deficient with respect to both plasma and bone zinc. Rats receiving 100 micrograms per day grew normally during the build-up period and had zinc levels within normal range in both plasma and bone. However, growth ceased very soon after the zinc was discontinued and by the end of the additional three weeks deficiency levels of zinc were observed in both plasma and bone. For animals receiving 1000 micrograms per day the bone zinc was markedly elevated and plasma zinc was temporarily above normal at the end of the build-up period. These rats continued to grow well through the additional three weeks, and although the zinc levels were decreasing they still maintained normal levels of plasma zinc and a good reserve in the bone.

Project Description (Continued):

A somewhat similar experiment was done in which animals were loaded as above with total amounts of 1000, 4900 and 8800 micrograms of zinc during the 10 day period then no further zinc was given, and growth and survival times were observed. Normal growth rates were observed in all groups during the 10 day build-up. Rats which received the 1000 micrograms ceased growing almost immediately and leveled off at 126 grams when zinc was discontinued. The other two groups continued normal growth for 28 and 42 days respectively after the zinc was withheld and leveled off at 180 and 224 grams. Gradual weight loss then followed with development of deficiency symptoms and finally death. The average survival time was lengthened as the zinc dosage was increased.

Similar experiments were done with germfree and ex-germfree rats. All animals were maintained germfree during the 10 day zinc loading period, then half were removed to a conventional environment. In general the results were comparable to the above experiments, and support the suggestion that bone zinc is indeed available for nutritional needs. In addition it was observed that at each zinc level (including zero-zinc) the germfree appear to be less deficient than the non-germfree, with respect to growth, onset of symptoms and survival times. It was also observed that female rats appear less deficient than males receiving similar amounts of zinc. The sex difference is more evident in germfree rats than in non-germfree. Some female rats which received 8800 micrograms of zinc during the 10 day loading have survived for peroids over 200 days without additional zinc. This suggests the requirement for zinc in the adult germfree rat may be extremely low compared to the requirement of a growing rat.

With Dr. Creveling we are attempting to produce animals with marked tryptophan deficiency or with abnormal tryptophan metabolism. He wishes to obtain suitable animal models in sufficient numbers to study alterations in the biochemistry of neuronal systems with respect to the unusual behavior of such animals. By nutritional means and within a period of about 30 days we have been able to produce rats with tryptophan dificiency of sufficient severity to be accompanied by peculiar behavior which may include hypersensitivity, tremors and seizures. A decreased level of 5-hydroxytryptamine, which is derived from tryptophan, has been observed in the tissues of these animals. We are also attempting to determine if diabetic rats, which have been shown to have abnormal tryptophan metabolism, may also exhibit any of these abnormalities.

ANNUAL REPORT SUMMARY

LABORATORY OF BIOCHEMISTRY AND METABOLISM

A. The Role of Hepatic Plasma Membranes in the Recognition and Catabolism of Circulating Glycoproteins. Continuing investigation into the mechanism whereby hepatic membranes bind and transport glycoproteins into the parenchymal cells may be summarized as follows:

1. In pursuit of the observation made last year that the hepatic membrane binding protein was present on the membranes of the smooth microsomes, the Golgi complex and the lysosomes, efforts have been directed toward elucidating the role of this specific binding protein in subcellular recognition phenomena. Toward this end, a number of membrane proteins have been isolated from the membranes of the various subcellular organelles which are recognized by the purified binding protein. Attempts to purify and characterize these receptor proteins are being currently investigated.

2. Together with Dr. Novogrodsky, at Cornell University, the purified rabbit liver membrane protein that binds desialylated glycoproteins has been shown to be a mitogen for human peripheral lymphocytes. The mitogenic activity is specific for desialylated T-cells. The potential relevance of this phenomenon to the etiology of hepatocellular necrosis is under consideration.

3. Based on the earlier identification of a mammalian binding protein specific for galactose-terminated proteins and the subsequent demonstration that avian species possessed an analogous protein with a specificity for N-acetylglucosamine-terminated proteins, the metabolism of serum glycoproteins was examined in fish. A series of modified glycoproteins with galactose-, N-acetylglucosamine and mannose termini were injected intravenously into fish. In no case was there rapid clearance from the circulation and no binding activity could be detected in the hepatic tissue, in vitro. It is tentatively concluded that this regulatory mechanism appeared subsequent to the evolution of fish and no further studies are planned.

B. The formation of the yeast primary septum. Work has continued on this topic in different areas, i.e., localization, solubilization and purification of chitin synthetase, topology of chitin in the yeast cell wall and bud scars and search for mutants in the chitin synthesis system. A new method for chitinase determination was also developed.

1. Localization, solubilization and purification of chitin synthetase. Previous results had shown that chitin synthetase was recovered in very good yield in a highly purified preparation of intact yeast plasma membranes. More recently Bracker et al. [Proc. Natl. Acad. Sci., USA 73: 4570-4574 (1976)] claimed that in extracts from the mold Mucor rouxii, the enzyme was associated with small particles, termed by those authors "chitosomes". In order to clarify whether such chitin synthetase-bearing particles were present in our preparation, the purified membranes were allowed to synthesize chitin for a short time. Calcofluor M-2R, a fluorescent brightener that binds

strongly to chitin was added and the preparation was examined under the fluorescence microscope. Bright spots appeared only over the membranes. This result was interpreted to show that nascent chitin was still probably attached to the sites where it was synthesized. Its distribution implied that chitin synthetase also was only present on the membranes and that it was distributed in many discrete areas rather than at a single spot, in confirmation of our previous hypothesis. These results are now being further tested and extended by electron microscope autoradiography.

The solubilization of the chitin synthetase zymogen paved the way for the further purification of the enzyme. In the first place, the extraction procedure was improved by first treating the membranes with cholate, that extracts many proteins and lipids but not chitin synthetase, and then with digitonin, which solubilizes the synthetase. The solubilized enzyme was further purified by chromatography on a Sepharose 6-B column, either before or after activation with trypsin. Overall purification, starting with the insoluble particles, was from 20 to 30-fold in different batches. The purified preparation still showed several bands on acrylamide gel electrophoresis in the presence of sodium dodecyl sulfate. Further purification of the enzyme by chromatography, using chitin as an affinity adsorbent is in progress.

The purified synthetase shows a requirement for a phospholipid. The best activators appear to be phosphatidyl-serine of lyso-phosphatidylserine. The activation effect depends on the digitonin concentration in the extracts and is under further study. The soluble synthetase is strongly inhibited by unsaturated, but not by saturated fatty acids.

2. Topology of chitin in the yeast cell wall and bud scars. As mentioned in a previous report, the topology of chitin in the bud scars was investigated by analyzing the distribution on the scar of ferritin-wheat germ agglutinin complexes in the electron microscope. This study was hampered by the apparent lack of specificity of the lectin. The agglutinin apparently binds to the whole surface of an alkali-digested cell envelope. Most of the non-specific binding is eliminated by previous incubation of the cell envelope with β-1,6-glucanase. More recently, it has been possible to obtain highly purified chitinase and use this protein, after conjugation with fluorescein, as a fluorescent probe for chitin. With the chitinase, fluorescence appears only at the bud scar site. Conjugates of chitinase with ferritin will be prepared and used for observations with the electron microscope. The effects previously obtained with wheat germ agglutinin are under further study, because of the potential information they may provide both on the cell wall structure and on the specificity of the lectin.

3. Search for mutants in the chitin synthetase system. A new screening procedure has been developed for mutants unable to synthesize chitin. After mutagenesis and growth of survivors on Petri plates, a replica of the colonies is made on a filter paper disc. The discs are dried at $37^{\circ}$, thus permeabilizing the cells. They are then incubated with a standard reaction mixture for chitin synthetase that includes $^{14}C$-labeled UDP-N-acetylglucosamine. After washing out the excess substrate on a funnel, the discs are dried and subjected to autoradiography. A wild-type

colony will give rise to a dark spot, corresponding to radioactive chitin, whereas at a mutant colony site either no spot or a lighter one will appear. With this procedure, several mutants have been isolated and are now under examination. Some strains with specific markers have been constructed to help in the genetic analysis of the mutants.

4. A chitinase assay. A very fast and sensitive method has been devised for chitinase determination. It involved the use of radioactive chitin as substrate. The chitin is prepared by reacetylation of chitosan with tritiated acetic anhydride and can be obtained at very high specific activities. After incubation, 10% trichloroacetic acid is added and the suspension is centrifuged. The reaction product, diacetylchitobiose, can be determined by simply counting the radioactivity of the supernatant.

The regenerated chitin, when prepared from chitosan with nonradioactive acetic anhydride, can also be used as an efficient adsorbent in the purification of chitinase or chitin synthetase.

Biosynthesis of a yeast glucan. Further studies were carried out on the in vitro synthesis of a β-1,3-linked polyglucose with a particulate preparation from Saccharomyces cerevisiae. The enzyme requires for maximal activity bovine serum albumin, glycerol and ATP. The latter can be partially substituted by a boiled extract from the same yeast (activating factor). It was found that most enzyme preparations lose rapidly their activity when incubated at 30°. This inactivation is increased by the presence of EDTA and decreased by boiled yeast extract (protecting factor). Fractionation of the extract by cationic exchange, barium precipitation and anionic exchange chromatography resulted in co-purification of both the activating and protecting factor. Inorganic polyphosphate was also associated with the active fractions. Recently, it has been found that an extract from baker's yeast had protecting but not activating ability. In this case, the protecting factor seems to be devoid of phosphate. Further studies of these materials are in progress.

A method for the detection of yeast nuclei. Staining of yeast and fungal nuclei by the Giemsa method is a long and cumbersome procedure of somewhat unpredictable outcome. An extremely fast and simple method was developed by using the antibiotic mithramycin, which binds tightly to DNA, giving rise to a strong fluorescence. The fluorescence can be observed immediately after mixing and the method is equally efficient for nuclei of yeast or filamentous fungi. In our case, the method will be useful to distinguish mutants blocked in nuclear division from those arrested in cytokinesis.

C. The Function of Membrane Protein Components in Bacteriocin Tolerance.

1. E. coli mutants tolerant to a specific set of colicins (tolF strains) have been characterized genetically. The tolF locus lies between pyrD and aroA on the E. coli chromosome. tolF strains show a marked reduction in the sensitivity to certain antibiotics such as tretracycline and polymyxin B.

2. tolF mutants examined are completely missing a single major outer membrane protein, protein Ia. This loss is nearly completely compensated for by an increase in one or two other major outer membrane proteins, proteins Ib and II*.

3. For a study of the physiological function of three major outer membrane proteins, Ia, Ib, II*, we have isolated a set of mutants specifically missing each of these proteins as well as all possible combinations of two or three Ia + Ib, etc.). Some of these mutants grow slowly on certain types of growth media, and we have found that in some cases the slow growth can be overcome by the addition of $Mg^{++}$ to the growth median.

4. A major outer membrane protein missing in tolG mutants has been purified from $tolG^{+}$ cells using preparative gel electrophoresis. As opposed to other methods of purification, use of this procedure leads to a nearly quantitative recovery of this protein. The purified material is homogeneous in three electrophoretic systems, contains 2 moles of reducing sugar/mole of peptide and has alanine as the N-terminal amino acid.

5. Escherichia coli ompA mutants are tolerant to colicin L-JF246. This tolerance can be overcome by a veriety of treatments that have as their target, the outer membrane or the peptidoglycan layers of the cell envelope. Thus, increasing the concentration of colicin, release of lipopolysaccharide from the outer membrane by treatment of intact cells with ethylenediaminetetracetic acid (EDTA), conversion of cells to spheroplasts by treatment with lysozyme-EDTA or penicillin, or trypsin treatment of intact cells, will result in an increased colicin sensitivity. These treatments alter or stretch the outer membrane or ompA mutants and suggest that the altered outer membrane may allow the penetration of at least a portion of the colicin molecule to a site of action located within this barrier. To substantiate this we have demonstrated that membrane visicles prepared from ompA mutants are sensitive to colicin L and that $^{14}C$-labelled colicin L binds rapidly to both the outer and inner membrane fractions of the cell.

D. Hormone-Dependent Differentiation of Mammary Gland.

1. The epithelium in the mammary gland of the adult male mouse resembles the anlage in the embryonic female. We have, therefore, used the male animal as a convenient source of embryonic-like mammary epithelium. It has been observed that appropriate hormone treatment in vivo can elicit growth and differentiation of the male epithelium equivalent to that which occurs in the pregnant female.

Using intact and hypophysectomized male mice, it has been determined that estrogen and prolactin are required for ductal growth. Alveolar growth requires progesterone, in addition.

Progesterone, although required for formation of alveoli, does not appear necessary for terminal differentiation of the ductal outgrowths resulting from stimulation by estrogen and prolactin. Thus, the ducts formed in

castrated, adrenalectomized male mice can synthesize α-lactalbumin in vitro in the presence of insulin, glucocorticoid and prolactin. Progesterone, then, may serve solely as a growth-promoter for mammary epithelium, with no positive function in its differentiation.

2. There is at least one important qualitative difference between mammary epithelium from primiparous non-pregnant and mature virgin mice. Only that from the virgin animals must traverse the cell-cycle in order to be able to synthesize milk-proteins in vitro in response to insulin, glucocorticoid and prolactin. Initial studies indicate the preventing such traversal has no detectable effect on ultrastructural development of the cells. But immunological analyses suggest that such blocked cells are not able to synthesize even the unphosphorylated polypeptide backbone of casein. Whether or not the corresponding m-RNA is transcribed under blocking conditions will be determined with the use of c-DNA.

3. We have reported that a modified form of insulin derived from insulin-Sepharose can elicit insulin-like effects in vitro from insulin-unresponsive tissues. Thus, whereas mammary epithelial cells from non-pregnant mice, and diaphragm from C57BL/$^{++}_{ob}$ $^{++}_{ob}$ mice do not respond to insulin, they do respond to the modified insulin. It was of interest to determine whether this difference in biological activity between the two forms of the hormone could also be demonstrated in vivo. Accordingly, liver ornithine decarboxylase (ODC) activity in the intact mouse has been studied. Insulin stimulates this activity 3-4 fold in the liver of lean C57BL mice, but has no stimulatory effect in C57BL/$^{++}_{ob}$ $^{++}_{ob}$ mice. The modified insulin does enhance ODC activity 3-4 fold in the liver of the obese, insulin-resistant mouse.

E. Spermidine and the development of Mammary Gland.

1. Studies on S-adenosyl-L-methionine decarboxylase and spermidine synthase, the two enzymes which are involved in the final step of spermidine biosynthesis, have shown that the activities of these two enzymes are stimulated by the actions of insulin and glucocorticoid. The decarboxylase has been purified to apparent homogeneity and its properties have been extensively studied. Using the pure form of the decarboxylase, we are developing the antibody for the enzyme, which is to be used for assessing the mechanism of the hormonal regulation of the decarboxylase.

2. To elucidate the mechanisms of action of spermidine in milk-protein synthesis at the molecular level, we have focused on the transcriptional and the translational control of casein mRNA. To this end, we examined the effect of glucocorticoid on the metabolism of casein mRNA in cultured mammary explants. These studies showed that glucocorticoid, in the presence of insulin, facilitates the accumulation of the mRNA, which remained untranslated without further exposure of mammary cells to prolactin. The results suggest the "two-step" regulation of milk-protein synthesis, one at the transcriptional and the other at the translational level. Glucocorticoid or spermidine appears to prolong the half-life of the untranslated casein mRNA.

F. Differentiation of Lymphoid Cells.

In order to study the means by which lipid A activates B cells but not T cells, it was first necessary to devise a procedure for labeling lipid A so that binding studies could be performed. An iodination technique was devised which provided a product having the appropriate chemical characteristics as well as the relative sensitivity that might be expected of an $^{125}I$-labeled indicator molecule. Such iodinated lipid A possesses the full mitogenic activity of untreated lipid A. Comparison of the $^{125}I$-lipid A binding activity of splenocytes and thymocytes from the same rabbit revealed that the extent of labeling of splenocytes was 10-20 times greater than that observed with an equivalent number of thymocytes. A similar preferential binding was detected in comparing cells in mouse and rat. Non-adherent spleen cell populations were essentially unaltered with regard to binding when compared to the original population. In addition, spleen cell populations enriched for T cells exhibited a marked loss of specific binding activity. On the other hand, spleen cell populations enriched for B cells exhibited the expected binding. The difference in binding behavior of B cell- and T cell-enriched populations was confirmed using three independent techniques to separate B cells and T cells. These findings are consistent with the mitogenic specificity of lipid A toward B cells rather than T cells and suggests that the observed cellular specificity resides in an early event in mitogenesis, i.e., binding of the mitogen.

At a concentration of $10^{-3}$ M a variety of nucleosides and nucleotides was found to have a pronounced effect on the induction of DNA synthesis by spleen and thymus cells incubated in tissue culture medium containing fetal calf serum. In some cases nucleosides and nucleotides were inhibitory and in others stimulatory. Spleen cells and thumus cells showed several differences in their behavior. For example, murine spleen cells cultured with GMP-5' exhibited enhanced DNA synthesis (about 3-fold), while the presence of AMP-5' during cell culture markedly inhibited DNA synthesis (>90%). On the other hand, murine thymus cells showed enhanced DNA synthesis in the presence of both AMP-5' and GMP-5' (5-fold). This disparity in behavior extends further in that DNA synthesis by rabbit thymus cells was inhibited by AMP-5', in contrast to findings with murine thymocytes. However, rabbit thymocytes yielded an enhanced thymidine incorporation in the presence of GMP-5'.

Although both AMP-5' and GMP-5' induce an increase in the intracellular levels of cyclic nucleotides, there was no consistent correlation in regard to the effects of AMP-5' and GMP-5' on DNA synthesis. Inhibition of DNA synthesis by AMP-5' appears to be an irreversible process and is selective in the sense that protein synthesis was essentially unaffected.

The fact that some nucleotides can be mitogenic as well as antimitogenic along with the commonly-held notion that phosphorous-containing compounds do not pass the cell membrane barrier, suggested the need for examining the distribution of plasma membrane-associated enzymes among the cells involved in the immune response as a prelude to understanding the possible function of ectoenzymes in the induction of DNA synthesis and immunoglobulin production.

It has been observed that rabbit spleen lymphocytes possess as much as 300 times the level of 5'-nucleotidase as found in an equivalent number of thymus cells. The enzyme activity in spleen populations is not principally associated with either T cells or B cells. Moreover, 5'-nucleotidase activity is not observed in bone marrow, appendix or buffy coat cell populations.

G. The Glutathione Transferases.

The glutathione S-transferases are among the major detoxication systems of the animal kingdom and perform this function by related mechanisms which all involve binding of protein and ligand. Our work with these enzymes has provided homogeneous transferases from human and rat liver. The availability of clean systems has allowed extensive studies on the chemical and kinetic mechanisms of the enzymatic reaction as well as the specificity of binding.

The glutathione S-transferases represent a family of enzymes with overlapping but broad specificity. By reason of their v-avidity in binding an enormous number of compounds bearing hydrophobic structure, they serve to passively hold compounds with highly electrophilic atoms in close proximity to glutathione. Indeed, the creation of this proximity effect may be their only function since the highly reactive nucleophile, glutathione thiolate, can then react with the first substrate. Because of their low rate enhancement and their relative lack of specificity these transferases appear to be primitive although effective enzymes.

Their spectrum of catalytic activity is revealed by the titles that have been bestowed on this group of enzymes. In current usage are the following terms: glutathione S-alkyltransferase; glutathione S-aryltransferase, glutathione S-aralkyltransferase, glutathione S-alkene transferase, glutathione S-epoxide transferase, nitrate ester reductase, steroid isomerase, $\Delta^5$-3-keto-steroid isomerase, maleylacetone isomerase, and ligandin. On the basis of our work with these catalytic activities in liver, it is probable that they should all be identified solely as glutathione S-transferase.

H. Biochemical Abnormalities Underlying Human Cystinosis.

Ten different fibroblast cell lines from patients with I-cell disease, an inherited lysosomal storage disorder unrelated to cystinosis, have been examined with respect to their intracellular cystine content. Of these lines, seven were found to have distinctly supra-normal levels (> 2 nanomoles half-cystine per mg protein) of this amino acid, one was marginally elevated, and two were normal (i.e., ca. 0.5 nanomoles/mg). Cystine levels from three cell lines from the biochemically similar, but clinically less severe variant of I-cell disease, viz., mucolipidosis III, were within normal limits, as were the levels in lines from patients with three different forms of mucopolysaccharide storage diseases.

I. Studies on Naturally Occurring Sulfur Nucleotides.

1. Search for a $S^4$U-free E. coli mutant. Such a mutant was reported by J. Jagger at U. Texas (Dallas) and obtained from him. It was found to be

lacking the first of the two $s^4U$-forming enzymes and normal with respect to the second. Large amounts of the $s^4U$-free tRNA are being produced to use with pure enzyme obtained from the present organism to study the reaction: kinetics, the reason for the ATP requirement, the nature of the product, and the mechanism for selecting only the U in position 8 of the tRNA for thiolation.

The parent and mutant organisms will also be compared for signs of differences in tRNA-related activites such as efficiency of cell wall biosynthesis and the size and composition of amino acid pools.

2. In a separate project, the new thionucleotide formed after λ infection of E. coli is studied. The modification represents new thiolation rather than further modification of a normally thiolated nucleotide in E. coli. The nucleotide has the properties of a purine and a $pK_a$ near 6.3. It is probably either methylated on a ring N or contains a secondary amino group in a side chain.

It is planned to continue with identification and to examine the distribution of the new nucleotide among the tRNA species in λ-infected E. coli. Dr. Adhya is mapping the location on λ, which involves testing tRNA in this laboratory, and we hope to work together on determining the role of the modification in λ infection.

J. Thermodynamic and Kinetic Studies of Protein Structure and Enzymic Mechanisms.

The variation of the rate of pepsinogen activation with pH has been reinvestigated. The results are interpreted to show that under mildly acidic conditions both uni- and bi-molecular mechanisms are rate limited by a rapid conformational change to an intermediate form of the zymogen. This change seems to involve separation of the positively charged amino terminal peptide from the rest of the molecule. This intermediate shows no proteolytic activity and does not bind pepsin substrates or inhibitors.

Conformational changes in pepsinogen at high pH have been investigated using kinetic methods. The molecule is destabilized by the presence of a buried positively charged group which does not titrate in the native protein, but is in slow equilibrium with a form where it titrates with pK = 6.75. Modification reactions suggest this group is a histidine residue.

The thermodynamics of unfolding of pepsinogen by protein denaturants are being investigated.

K. Regulation of Glycolysis in Brain.

Brain, primarily a glycolytic organ, has been shown to contain $FruP_2$ase, a key enzyme in gluconeogenesis. Previous attempts elsewhere to demonstrate the enzyme in brain have been unsuccessful. Although the amount of enzyme is comparable to that in liver the specific activity is only 1/200 of the

liver enzyme, a fact which may account for the low gluconeogenic activity of brain. The enzyme has been purified 300-fold to 95% homogeneity and is virtually specific for $FruP_2$, no other bisphosphate or monophosphate serving as substrate except sedoheptulose 1,7-bisphosphate toward which the enzyme is 1/5 as active. $K_m$ for $FruP_2$ = 1.32 x $10^{-4}$ M. In contrast to other mammalian $FruP_2$ases, brain $FruP_2$ase has no metal requirement and is not inhibited by 5'-AMP or EDTA; it is maximally active above pH 7.6. The enzyme cross reacts with its own antibody but not with antibody to the liver enzyme, showing differences between the two enzymes although they both have the same subunit molecular weight of 40,000. The enzyme is quantitatively precipitated by its antibody and the precipitated complex is enzymatically active. The enzyme is not stimulated by phospholipid and, therefore, cannot account for the inhibition of glycolysis in brain by phospholipid reported previously.

L. Particulate Enzymes in Carbohydrate Metabolism.

Glucose-6-phosphatase and its associated $PP_i$-glucose phosphotransferase activity, NADPH-cytochrome c reductase, nucleoside diphosphatase and S-palmitoyl-coenzyme A:sn-glycerol-3-phosphate acyltransferase were studied in rats as a function of the age of the animals. These enzymes were selected because of their location within the membrane and their possible role in the synthesis of membrane components or importance to the development of metabolism. Since glucose-6-phosphatase and nucleoside diphosphatase have latent activities which can be expressed by agents which disrupt membranes, we have also studied age-related changes in the latencies of these activities.

Results for rat liver indicate the existence of several distinct patterns of enzyme development. Glucose-6-phosphatase of liver increased rapidly after birth to a level several-fold higher than that in adult liver and declined with age, while nucleoside diphosphatase gradually increased to reach adult levels. NADPH-cytochrome c reductase and S-palmitoyl-coenzyme A:sn-glycerol-3-phosphate acyltransferase underwent essentially parallel changes in activity with age, increasing and decreasing during the neonatal period before increasing again to adult levels during the weaning period.

The development of glucose-6-phosphatase in the kidney was found to be strikingly different from that in the liver. Both the hydrolase and the phosphotransferase activities, which were very low at birth, underwent a small increase and decrease in the neonatal kidney followed by a gradual increase in activity. The same activities in liver fell rapidly from the very high levels reached a few days after birth until the kidney and liver enzymes were at approximately the same levels in adult animals. In contrast, the other three enzymes underwent approximately parallel age-related changes in kidney and liver with the absolute levels remaining lower in kidney.

During the neonatal period all the enzymes studied had higher activities in rough than in smooth liver microsomes. Glucose-6-phosphatase differed from the other enzymes in exhibiting non-parallel changes with age in rough and smooth membranes. Glucose-6-phosphatase activity of rough membranes increased greatly during the first few days of life, while the activity in smooth membranes showed no such neonatal overshoot. Results indicate that

there is no general pattern of acquisition for membrane-bound enzymes of proliferating ER membranes. Clearly the development of these enzymes is more closely related to the changing metabolic requirements of the animal than to membrane proliferation.

The degree of latency of glucose-6-phosphate phosphohydrolase has been found to be very much greater in the neonatal than in the adult liver. In contrast, the transferase function of the enzyme retains a relatively high degree of latency throughout life. In this regard it resembles nucleoside diphosphatase which is known to have a luminal location on the microsomal membrane. Since the location of the active sites of glucose-6-phosphatase, a phospholipid-requiring enzyme which is probably an integral part of the endoplasmic reticular membrane, has been the subject of a number of conflicting hypotheses, we propose to extend our latency studies to include the relative effectiveness of proteolytic enzymes and the effects of varying concentrations of detergents which are known to change membrane permeability. An attempt will be made to determine if the observed properties can be explained by a transmembrane localization of the enzyme molecule or if the existence of a specific substrate transport mechanism must be evoked.

M. Biochemical Lesions in Genetic Mucopolysaccharidosis.

1. The isolation and tritiation of the basic disulfated disaccharide substrate have been significantly improved. Previously from 5-10% of the isolated disaccharide was resistant to borohydride reduction. Modification of the procedure results in complete reduction of the anhydromannose moiety. Samples of the substrate have been made available to laboratories in the United States and abroad.

2. Iduronate sulfatase substrate of very high specific activity has been prepared and applied to the detection of the heterozygous state by assaying the enzymatic activity in hair root follicles.

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PERIOD COVERED
July 1, 1976 through September 30, 1977

TITLE OF PROJECT (80 characters or less)
The Role of Hepatic Plasma Membranes in the Turnover of Circulating Glycoproteins.

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

| | | | |
|---|---|---|---|
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| Other: | Jean Hickman | Research Chemist | LBM NIAMDD |
| | William Pricer | Research Chemist | LBM NIAMDD |
| | Tadashi Tanabe | Visiting Scientist | LBM NIAMDD |

COOPERATING UNITS (if any)
None

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SECTION
Section on Enzymes and Cellular Biochemistry

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20014

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

A variety of subcellular glycoproteins have been identified as recognition markers for the isolated hepatic binding protein. The role of these proteins is being studied as a key to the mechanism of pinocytosis, an example of a well-defined intracellular recognition event.

The same hepatic binding protein has been shown to be a mitogen for desialylated lymphocytes (T-cells) and the relevance of this phenomena to the etiology of hepatocellular necrosis is being pursued.

Extension of the above studies to human liver has been undertaken in an attempt to characterize the pathological changes taking place in the hepatic binding protein which results in elevated serum levels of asialoglycoproteins in patients with cirrhosis, hepatitis and hepatoma.



PHS-6040
(Rev. 10-76)

Project Description:

The Role of Hepatic Plasma Membranes in The Recognition and Catabolism of Circulating Glycoproteins. Continuing investigation into the mechanism whereby hepatic membranes bind and transport glycoproteins into the parenchymal cells may be summarized as follows:

1. In pursuit of the observation made last year that the hepatic membrane binding protein was present on the membranes of the smooth microsomes, the Golgi complex and the lysosomes, efforts have been directed toward elucidating the role of this specific binding protein in subcellular recognition phenomena. Toward this end, a number of membrane proteins have been isolated from the membranes of the various subcellular organelles which are recognized by the purified binding protein. Attempts to purify and characterize these receptor proteins are being currently investigated.

2. Together with Dr. Novogrodsky, at Cornell University, the purified rabbit liver membrane protein that binds desialylated glycoproteins has been shown to be a mitogen for human peripheral lymphocytes. The mitogenic activity is specific for desialylated T-cells. The potential relevance of this phenomenon to the etiology of hepatocellular necrosis is under consideration.

3. Based on the earlier identification of a mammalian binding protein specific for galactose-terminated proteins and the subsequent demonstration that avian species possessed an analogous protein with a specificity for N-acetylglucosamine-terminated proteins, the metabolism of serum glycoproteins was examined in fish. A series of modified glycoproteins with galactose-, N-acetylglucosamine-, and mannose termini were injected intravenously into fish. In no case was there rapid clearance from the circulation and no binding activity could be detected in the hepatic tissue, in vitro. It is tentatively concluded that this regulatory mechanism appeared subsequent to the evolution of fish and no further studies are planned.

Proposed Course of Project:

1. It is proposed to continue the studies in progress on the isolation and characterization of receptors on the membranes of hepatic subcellular organelles as part of a study of the mechanism of pinocytosis. In addition, a kinetic study of the time sequence in which the mammalian binding protein appears in the various loci will be undertaken to determine the biogenetic origin of the lysosomal protein.

2. With the previous demonstration that patients with hepatic pathology (cirrhosis, hepatitis, hepatoma) exhibit elevated levels of serum asialoglycoproteins, the isolation and characterization of the human binding protein will be undertaken. Antibody to this material will be used as a probe to study the pathological changes in the diseased state.

Publications:

Kawasaki, T. and Ashwell, G.: Carbohydrate structure of glycopeptides isolated from an hepatic membrane—binding protein specific for asialoglycoproteins. J. Biol. Chem. 251: 5292-5299. 1976.

Pricer, W. E. and Ashwell, G.: Subcellular distribution of a mammalian hepatic binding protein specific for asialoglycoproteins. J. Biol. Chem. 251: 7539-7544, 1976.

Novogrodsky, A. and Ashwell, G.: Lymphocyte mitogenesis induced by a mammalian liver protein that specifically binds desialylated glycoproteins. Proc. Nat. Acad. Sci., 74: 676-678, 1977.

Ashwell, G. and Morell, A. G.: Membrane glycoproteins and recognition phenomena. Trends Biochem. Sci. 2: 76-78, 1977.

Ashwell, G.: The role of cell-surface carbohydrates in binding phenomena. In Jamieson, G. A. and Robinson, D. M. (Ed.): Mammalian Cell Membranes. London-Boston, Butterworths, 1977, pp. 57-71.

Kawasaki, T. and Ashwell, G.: The isolation and characterization of an avian hepatic binding protein specific for N-Acetylglucosamine-terminated glycoproteins. J. Biol. Chem., in press.

Ashwell, G.: A functional role for lectins. Trends Biochem. Sci., in press.

| SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space) | U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT | PROJECT NUMBER Z01 AM 17002-07 LBM |
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PERIOD COVERED
July 1, 1976 through September 30, 1977

TITLE OF PROJECT (80 characters or less)

Catalysis by Glutathione Transferases

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

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| Other: | James M. Keen | Staff Fellow | LBM NIAMDD |
| | Ronald Sekura | Staff Fellow | LBM NIAMDD |
| | Richard Weisiger | Research Associate | LBM NIAMDD |
| | Carol Marcus | Guest Worker | LBM NIAMDD |

COOPERATING UNITS (if any)

None

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The glutathione S-transferases appear to have three functions: They serve as catalysts in reactions in which glutathione is used to attack a vast number of compounds with a susceptible electrophilic atom; they serve as binding proteins for an even larger number of compounds that include bilirubin and heme; they serve as one of the reactants in the formation covalent complexes with very highly reactive reagents including those with carcinogenic activity. All three functions have been investigated but that of catalysis has been emphasized.



PHS-6040
(Rev. 10-76)

Project Description:

The glutathione S-transferases are among the major detoxification systems of the animal kingdom and perform this function by related mechanisms which all involve binding of protein and ligand. Our work with these enzymes has provided homogeneous transferases from human and rat liver. The availability of clean systems has allowed extensive studies on the chemical and kinetic mechanisms of the enzymatic reaction as well as the specificity of binding.

The glutathione S-transferases represent a family of enzymes with overlapping but broad specificity. By reason of their avidity in binding an enormous number of compounds bearing hydrophobic structure, they serve to passively hold compounds with highly electrophilic atoms in close proximity to glutathione. Indeed, the creation of this proximity effect may be their only function since the highly reactive nucleophile, glutathione thiolate, can then react with the first substrate. Because of their low rate enhancement and their relative lack of specificity these transferases appear to be primitive although effective enzymes.

Their spectrum of catalytic activity is revealed by the titles that have been bestowed on this group of enzymes. In current usage are the following terms: glutathione S-alkyltransferase; glutathione S-aryltransferase, glutathione S-aralkyltransferase, glutathione S-alkene transferase, glutathione S-epoxide transferase, nitrate ester reductase, steroid isomerase, $\Delta^5$-3-keto-steroid isomerase, maleylacetone isomerase, and ligandin. On the basis of our work with these catalytic activities in liver, it is probable that they should all be identified solely as glutathione S-transferase.

Proposed Course of Project:

Work on glutathione conjugation by enzymes from liver is intimately related to the general mechanism of aromatic oxidation as well as to the biological problem of detoxication of noxious compounds. It is expected that investigations dealing with the above-noted projects will be continued and a study of other conjugation reactions will be initiated.

Publications:

Keen, J. H., Habig, W. H., and Jakoby, W. B.: A mechanism for the several activities of the glutathione S-transferases. J. Biol. Chem. 251: 6183-6188, 1976.

Wolkoff, A. W., Ketley, J. N., Waggoner, J. G., Jakoby, W. B., and Berk, P. D.: Hepatic storage and intracellular binding of conjugated bilirubin. Gastroenterology 71: 936, 1976.

Jakoby, W. B.: The glutathione S-transferases: A triple-threat in detoxication. In deSerres, F., Fonts, J. R., Bend, J. R., and Philpat, R. M., (Eds.):

In Vitro Metabolic Activation in Mutagenesis Testing. Amsterdam, North Holland Press, 1976, pp. 207-212.

Habig, W. H., Pabst, M. J., and Jakoby, W. B.: Glutathione S-transferase AA from rat liver. Arch. Biochem. Biophys. 175: 710-716, 1976.

Benson, A. M., Talalay, P., Keen, J. H., and Jakoby, W. B.: Relationship between the soluble glutathione-dependent $\Delta^5$-3-ketosteroid isomerase and the glutathione S-transferases of the liver. Proc. Nat. Acad. Sci., USA 74: 158-162, 1977.

Jakoby, W. B.: The glutathione S-transferases: A group of multifunctional detoxification proteins. Adv. Enzymol. 46: 381-412, 1977.

Jakoby, W. B. and Wilchek, M. (Eds.): Methods in Enzymology. 46. Affinity Labeling. New York, Academic Press, 1977, 760 pp.

Berk, P. D. Wolkoff, A. W., Scharschmidt, B. R., Shupeck, M., Ketley, J. N., Waggoner, J. G., and Jakoby, W. B.: Recent studies of the metabolism of conjugated bilirubin. In Schaffner, F., Bianchi, L., Gerok, W., and Sickinger, K. (Eds.): Liver and Bile. Lancaster, MTP Press, 1977, in press.

Jakoby, W. B.: Glutathione S-transferases: A family of binding proteins which includes ligandin. In Berk, P. D., Berlin, N., and Watson, C. (Eds.): Chemistry and Metabolism of Bile Pigments. Washington, D.C., U.S. Government Printing Office, in press.

Jakoby, W. B., and Keen, J. H.: A triple-threat in detoxification: The glutathione S-transferases. Trends Biochem. Sci., in press.

Pinkus, L. M., Ketley, J. N., and Jakoby, W. B.: The glutathione S-transferases as a possible detoxification system of rat intestinal epithelium. Biochem. Pharmacol., in press.

| SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space) | U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT** | PROJECT NUMBER Z01 AM 17003-10 LBM |
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PERIOD COVERED

July 1, 1976 - September 30, 1977

TITLE OF PROJECT (80 characters or less)

Polysaccharides in Morphogenesis

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

| | | | |
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| OTHER: | Angel Duran | Visiting Associate | LBM NIAMDD |
| | Jesus Molano | Visiting Fellow | LBM NIAMDD |
| | Eleanor Shematek | Staff Fellow | LBM NIAMDD |
| | Martin Slater | Staff Fellow | LBM NIAMDD |

COOPERATING UNITS (if any)

Blair Bowers, LCB, NHLI

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The distribution of chitin synthetase on the yeast plasma membrane will be further studied by electron microscopy and autoradiography. Purification of the solubilized synthetase will be continued with an electrophoretically homogeneous preparation as a goal. Samples of chitin made by yeast in vivo or synthesized in vitro using plasma membranes or solubilized enzyme will be compared, to ascertain how much spatial organization, if any, of the enzyme molecules is necessary to obtain a product identical to the "in vivo" polysaccharide. Mutants defective in chitin synthesis will be studied genetically and biochemically, to ascertain the nature of their defect.

The topology of chitin distribution in the yeast cell wall will be examined by electron microscopy, using ferritin-chitinase conjugates.

The factors responsible for the protection and activation of a β-1,3-glucan synthetase will be subjected to further studies.

Project Description:

Objectives - The ongoing research attempts to provide an understanding of the molecular mechanisms of morphogenesis. The topics currently under study are the formation of the primary septum of yeast and the biosynthesis of a glucan which is a major structural component of the yeast cell wall.

Methods Employed - The organism used for these studies is Saccharomyces cerevisiae, grown in the laboratory. Appropriate strains are available for different purposes, including strains with specific markers. Mutants are obtained by mutagenesis with either nitrosoguanidine or ethyl methyl sulfonate and screened as indicated under "Major Findings".

Cells are converted into protoplasts by treatment with snail intestinal juice. Protoplasts are disrupted by osmotic lysis and mechanical shearing to obtain particulate fractions. The latter are further fractionated by centrifugation in density gradients. Enzymes are solubilized with detergents and purified by conventional methods.

Proteins used in studies of chitin localization are conjugated with either fluorescein or ferritin for studies by fluorescence or electron microscopy, respectively. Glutaraldehyde or an isothio-cyante are used as condensing agents.

Major Findings - The formation of the yeast primary septum. Work has continued on this topic in different areas, i.e., localization, solubilization and purification of chitin synthetase, topology of chitin in the yeast cell wall and bud scars and search for mutants in the chitin synthesis system. A new method for chitinase determination was also developed.

a) Localization, solubilization and purification of chitin synthetase. Previous results had shown that chitin synthetase was recovered in very good yield in a highly purified preparation of intact yeast plasma membranes. More recently Bracker et al. [Proc. Natl. Acad. Sci. USA 73:4570-4574, (1976)] claimed that in extracts from the mold Mucor rouxii, the enzyme was associated with small particles, termed by those authors "chitosomes". In order to clarify whether such chitin synthetase-bearing particles were present in our preparation, the purified membranes were allowed to synthetize chitin for a short time. Calcofluor M-2R, a fluorescent brightener that binds strongly to chitin was added and the preparation was examined under the fluorescence microscope. Bright spots appeared only over the membranes. This result was interpreted to show that nascent chitin was still probably attached to the sites where it was synthesized. Its distribution implied that chitin synthetase also was only present on the membranes and that it was distributed in many discrete areas rather

than at a single spot, in confirmation of our previous hypothesis. These results are now being further tested and extended by electron microscope autoradiography.

The solubilization of the chitin synthetase zymogen paved the way for the further purification of the enzyme. In the first place, the extraction procedure was improved by first treating the membranes with cholate, that extracts many proteins and lipids but not chitin synthetase, and then with digitonin, which solubilizes the synthetase. The solubilized enzyme was further purified by chromatography on a Sepharose 6-B column, either before or after activation with trypsin. Overall purification, starting with the insoluble particles, was from 20 to 30-fold in different batches. The purified preparation still showed several bands on acrylamide gel electrophoresis in the presence of sodium dodecyl sulfate. Further purification of the enzyme by chromatography, using chitin as an affinity adsorbent is in progress.

The purified synthetase shows a requirement for a phospholipid. The best activators appear to be phosphatidyl-serine or lyso-phosphatidylserine. The activation effect depends on the digitonin concentration in the extracts and is under further study. The soluble synthetase is strongly inhibited by unsaturated, but not by saturated fatty acids.

b) Topology of chitin in the yeast cell wall and bud scars. As mentioned in a previous report, the topology of chitin in the bud scars was investigated by analyzing the distribution on the scar of ferritin-wheat germ agglutinin complexes in the electron microscope. This study was hampered by the apparent lack of specificity of the lectin. The agglutinin apparently binds to the whole surface of an alkali-digested cell envelope. Most of the non-specific binding is eliminated by previous incubation of the cell envelope with β-1,6-glucanase. More recently it has been possible to obtain highly purified chitinase and use this protein, after conjugation with fluorescein, as a fluorescent probe for chitin. With the chitinase, fluorescence appears only at the bud scar site. Conjugates of chitinase with ferritin will be prepared and used for observations with the electron microscope. The effects previously obtained with wheat germ agglutinin are under further study, because of the potential information they may provide both on the cell wall structure and on the specificity of the lectin.

c) Search for mutants in the chitin synthetase system. A new screening procedure has been developed for mutants unable to synthesize chitin. After mutagenesis and growth of survivors on Petri plates, a replica of the colonies is made on a filter paper disc. The discs are dried at 37°, thus permeabilizing the cells. They are then incubated with a standard reaction mixture for chitin synthetase that includes $^{14}C$-labeled UDP-N-acetylglucosamine. After washing out the excess substrate on a funnel, the discs are dried and subjected to autoradiography. A wild-type

colony will give rise to a dark spot, corresponding to radioactive chitin, whereas at a mutant colony site either no spot or a lighter one will appear. With this procedure, several mutants have been isolated and are now under examination. Some strains with specific markers have been constructed to help in the genetic analysis of the mutants.

d) A chitinase assay - A very fast and sensitive method has been devised for chitinase determination. It involved the use of radioactive chitin as substrate. The chitin is prepared by reacetylation of chitosan with tritiated acetic anhydride and can be obtained at very high specific activities. After incubation, 10% trichloroacetic acid is added and the suspension is centrifuged. The reaction product, diacetylchitobiose, can be determined by simply counting the radioactivity of the supernatant.

The regenerated chitin, when prepared from chitosan with nonradioactive acetic anhydride, can also be used as an efficient adsorbent in the purification of chitinase or chitin synthetase.

Biosynthesis of a yeast glucan - Further studies were carried out on the in vitro synthesis of a β-1,3-linked polyglucose with a particulate preparation from Saccharomyces cerevisiae. The enzyme requires for maximal activity bovine serum albumin, glycerol and ATP. The latter can be partially substituted by a boiled extract from the same yeast (activating factor). It was found that most enzyme preparations lose rapidly their activity when incubated at 30°. This inactivation is increased by the presence of EDTA and decreased by boiled yeast extract (protecting factor). Fractionation of the extract by cationic exchange, barium precipitation and anionic exchange chromatography resulted in co-purification of both the activating and the protecting factor. Inorganic polyphosphate was also associated with the active fractions. Recently, it has been found that an extract from baker's yeast had protecting but not activating ability. In this case, the protecting factor seems to be devoid of phosphate. Further studies of these materials are in progress.

A method for the detection of yeast nuclei. Staining of yeast and fungal nuclei by the Giemsa method is a long and cumbersome procedure of somewhat unpredictable outcome. An extremely fast and simple method was developed by using the antibiotic mithramycin, which binds tightly to DNA, giving rise to a strong fluorescence. The fluorescence can be observed immediately after mixing and the method is equally efficient for nuclei of yeast or filamentous fungi. In our case, the method will be useful to distinguish mutants blocked in nuclear division from those arrested in cytokinesis.

Significance to Bio-Medical Research and the Program of the Institute:

The ongoing and proposed work deals with fundamental processes of differentiation and morphogenesis that occur in all living cells. More knowledge about such basic processes is essential for our understanding of cellular function both in health and in disease.

Proposed Course of Project:

See Summary.

Publications:

Cabib, E.: The yeast primary septum: a journey into three-dimensional biochemistry. Trends in Biochem. Sci. 1: 275-277, 1976.

| SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space) | U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT** | PROJECT NUMBER Z01 AM 17004-09 LBM |
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PERIOD COVERED

July 1, 1976 - September 30, 1977

TITLE OF PROJECT (80 characters or less)

Thermodynamic and Kinetic Studies of Protein Structure and Enzymic Mechanisms

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT


| | | | |
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| OTHER: | Faizan Ahmad | Visiting Fellow | LBM NIAMDD |


COOPERATING UNITS (if any)

LAB/BRANCH

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)


Studies on the relationship between protein conformation and enzymic activity are to be continued. Most specifically, we are interested in the changes in conformation and intramolecular interactions accompanying the transformation of pepsinogen to pepsin. These changes produce an enormous increase in enzymic activity and in the relative stability of the molecules.




PHS-6040
(Rev. 10-76)

Project Description:

Objectives - The digestive enzyme, pepsin is synthesized as an inactive zymogen, pepsinogen. It is hoped that studies on the conformation of these two proteins will reveal the mechanism by which proteolytic activity is repressed in the zymogen.

Methods Employed - Visible and Ultraviolet spectroscopy; rapid kinetic experiments in Aminco T-jump and stop flow apparatus. These have recently been equipped with a Dasar system which greatly facilitates data collection and processing through an interface with the TSO computing system.

Major Findings - The variation of the rate of pepsinogen activation with pH has been reinvestigated. The results are interpreted to show that under mildly acidic conditions both uni- and bi-molecular mechanisms are rate limited by a rapid conformational change to an intermediate form of the zymogen. This change seems to involve separation of the positively charged amino terminal peptide from the rest of the molecule. This intermediate shows no proteolytic activity and does not bind pepsin substrates or inhibitors.

Conformational changes in pepsinogen at high pH have been investigated using kinetic methods. The molecule is destabilized by the presence of a buried positively charged group which does not titrate in the native protein, but is in slow equilibrium with a form where it titrates with pK = 6.75. Modification reactions suggest this group is a histidine residue.

The thermodynamics of unfolding of pepsinogen by protein denaturants are being investigated.

Proposed Course of Project - Studies on the mechanism of activation of pepsinogen to pepsin are being continued. By the use of synthetic inhibitors chemical modification and stability studies, it is hoped to reveal more about the differences in conformation between these two proteins and the basis of activity in the derived enzyme.

Publications:

McPhie, P.: A turbidimetric milk clotting assay for pepsin. Anal. Biochem. 73: 258-261, 1977.

McPhie, P.: On the apparent inhibition of pepsinogen activation by pepsin substrates. J. Biol. Chem. (in press).

Bergeron, R. J. and McPhie, P.: Circular dichroism studies on the structure of p-nitrophenolate cycloamylose complexes. Bioorganic Chemistry (in press).

| SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space) | U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT | PROJECT NUMBER Z01 AM 17005-12 LBM |
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PERIOD COVERED
July 1, 1976 through September 30, 1977

TITLE OF PROJECT (80 characters or less)
Studies on Naturally Occurring Sulfur Nucleotides: Identification, Chemistry, Biosynthesis and Biological Significance

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT


PI: Marie N. Lipsett Research Chemist LBM NIAMDD
OTHER: Joseph D. Cherayil Visiting Associate LBM NIAMDD


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Sankar L. Adhya, LMBG4, NCI

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)


This project encompasses identification of thionucleotides in tRNA and studies of their enzymatic syntheses and their roles in modifying such tRNA activities as regulation of amino acid levels and protein synthesis.

The present work includes: 1) A study of the first of two enzymes forming 4-thiouridine ($s^4U$) in tRNA with respect to kinetics, the reason for the ATP requirement and the nature of the product. The enzyme is pure, the tRNA substrate will be obtained from an E. coli mutant lacking $s^4U$; 2) A study of the amino acid pool size and composition in this $s^4U$-deficient mutant and its parent, to determine whether $s^4U$ influences this tRNA regulatory function; and 3) (with S.L. Adhya) Identification and study of a new thionucleotide arising in E. coli after infection with bacteriophage λ.





PHS-6040
(Rev. 10-76)

Major Findings and Proposed Course of Work:

1) Search for a $s^4U$-free E. coli mutant. In the course of my own search, such a mutant was reported by J. Jagger at U. Texas (Dallas) and obtained from him. It was found to be lacking the first of the two $s^4U$-forming enzymes and normal with respect to the second. Large amounts of the $s^4U$-free tRNA are being produced to use with pure enzyme obtained from the parent organism to study the reaction: kinetics, the reason for the ATP requirement, the nature of the product, and the mechanism for selecting only the U in position 8 of the tRNA for thiolation.

The parent and mutant organisms will also be compared for signs of differences in tRNA-related activities such as efficiency of cell wall biosynthesis and the size and composition of amino acid pools.

2) In a separate project, started with SLA and JDC before the latter returned to India in mid-July 1976, the new thionucleotide formed after λ infection of E. coli is studied. The modification represents new thiolation rather than further modification of a normally thiolated nucleotide in E. coli. The nucleotide has the properties of a purine and a $pK_a$ near 6.3. It is probably either methylated on a ring N or contains a secondary amino group in a side chain.

It is planned to continue with identification and to examine the distribution of the new nucleotide among the tRNA species in λ-infected E. coli. Dr. Adhya is mapping the location on λ, which involves testing tRNA in this laboratory, and we hope to work together on determining the role of the modification in λ infection.

Publications:

Cherayil, J. S. and Lipsett, M. N.: Zeatin Ribonucleosides in the Transfer Ribonucleic Acid of Rhizobium leguminosarum, Agrobacterium tumefaciens, Corynebacterium fascians and Erwinia amylovora. J. Bacteriol., in press.

| SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space) | U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT | PROJECT NUMBER ZO1 AM 17006-03 LBM |
|---|---|---|

PERIOD COVERED
July 1, 1976 through September 30, 1977

TITLE OF PROJECT (80 characters or less)
Function of Membrane Protein Components in Bacteriocin Tolerance

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT


| | | | |
|---|---|---|---|
| PI: | John F. Foulds | Research Chemist | LBM NIAMDD |
| OTHER: | Tuu-jyi Chai | Staff Fellow | LBM NIAMDD |


COOPERATING UNITS (if any)
None

LAB/BRANCH
Laboratory of Biochemistry and Metabolism

SECTION
Section on Enzymes and Cellular Biochemistry

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20014

| TOTAL MANYEARS: | PROFESSIONAL: | OTHER: |
|---|---|---|
| 2 | 2 | 0 |

SUMMARY OF WORK (200 words or less - underline keywords)


The cell envelope of Escherichia coli is usually described as a 3-layered structure. The outermost layer, the outer membrane, is composed primarily of phospholipids, lipopolysaccharide and proteins. The proteins are distinguished in that a few (4-6) major proteins with molecular weights between 7,800 and 40,000 account for about 70% of the total protein found in the outer membrane.

Our goal is to describe the means whereby the major outer membrane proteins are synthesized and translocated specifically to this membrane and to define their physiological function.

We have isolated mutants missing as many as 4 of these major outer membrane proteins by selection of colicin-tolerant and/or bacteriophage-resistant cells. For example, outer membrane materials prepared from tolF mutants lack a specific major outer membrane protein, protein Ia. The loss of this protein is almost completely compensated for by the increased levels of one or two other major outer membrane proteins. Analysis of mutants missing protein Ia has revealed there are several genetic loci (at least 4) concerned with the expression of outer membrane protein Ia. Studies of these loci should provide information on the regulation of synthesis of protein Ia.




PHS-6040
(12-75)

Project Description:

Objectives:

The goal of this project is to understand some questions of general biological interest:

1. What is the function of the major outer membrane proteins?

2. Where are they synthesized?

3. How are they translocated to the outer membrane?

4. Why are they found primarily (exclusively?) in the outer membrane?

5. Can the absence of some outer membrane proteins be compensated for by mechanisms such as increased levels of other proteins?

6. What is the relationship between the structure of these proteins and their function?

Major Findings:

1. E. coli mutants tolerant to a specific set of colicins (tolF strains) have been characterized genetically. The tolF locus lies between pyrD and aroA on the E. coli chromosome. tolF strains show a marked reduction in the sensitivity to certain antibiotics such as tetracycline and polymyxin B.

2. All tolF mutants examined are completely missing a single major outer membrane protein, protein Ia. This loss is nearly completely compensated for by an increase in one or two other major outer membrane proteins, proteins Ib and $II^*$.

3. For a study of the physiological function of three major outer membrane proteins, Ia, Ib, $II^*$, we have isolated a set of mutants specifically missing each of these proteins as well as all possible combinations of two or three (Ia + Ib, etc.). Some of these mutants grow slowly on certain types of growth media, and we have found that in some cases the slow growth can be overcome by the addition of $Mg^{++}$ to the growth median.

4. A major outer membrane protein missing in tolG mutants has been purified from $tolG^+$ cells using preparative gel electrophoresis. As opposed to other methods of purification, use of this procedure leads to a nearly quantitative recovery of this protein. The purified material is homogeneous in three electrophoretic systems, contains 2 moles of reducing sugar/mole of peptide and has alanine as the N-terminal amino acid.

5. Escherichia coli ompA mutants are tolerant to colicin L-JF246. This tolerance can be overcome by a variety of treatments that have as their target, the outer membrane or the peptidoglycan layers of the cell envelope. Thus, increasing the concentration of colicin, release of lipopolysaccharide from the outer membrane by treatment of intact cells with ethylenediaminetetracetic acid (EDTA), conversion of cells to spheroplasts by treatment with lysozyme-EDTA or penicillin, or trypsin treatment of intact cells, will result in an increased colicin sensitivity. These treatments alter or stretch the outer membrane or ompA mutants and suggest that the altered outer membrane may allow the penetration of at least a portion of the colicin molecule to a site of action located within this barrier. To substantiate this we have demonstrated that membrane vesicles prepared from ompA mutants are sensitive to colicin L and that $^{14}$C-labelled colicin L binds rapidly to both the outer and inner membrane fractions of the cell. (This material has been submitted to the Proc. Natl. Acad. Sci., U.S.A.).

6. Collaborative studies

a. With C. Raetz. Mutants of Escherichia coli K-12 defective in phosphatidylserine synthetase (pss) can be isolated as temperature-sensitive, conditional lethals. When cultivated at intermediate temperatures (30°), such mutants contain approximately three times more phosphatidylglycerol plus cardiolipin (and less phosphatidylethanolamine) than normal. We found that under these conditions the pss8 mutant is hypersensitive to certain antibiotics, particularly streptomycin, ampicillin, gentamycin and novobiocin. This suggests a markedly increased permeability of the outer membrane. However, the total membrane protein and fatty acid composition is nearly normal, and bacteriophage growth studies demonstrate that lipopolysaccharide is functionally intact. These results prove that the antibiotic hypersensitivity of pss8 differs from other hypersensitivities reported previously, and suggest that a putative inhibitor of phosphatidylserine synthetase would greatly potentiate the action of numerous antibiotics currently in clinical use.

b. With M. Deutscher. We have investigated the role of tRNA nucleotidyltransferase in tRNA metabolism using mutants (CCA mutants) of E. coli deficient in this enzyme. Extracts of the most defective strain contained less than 2% the normal level of enzyme. Such mutants grew very slowly, even under optimal growth conditions and about 10% of the tRNA isolated from these cells contained complete termini. The slow growth of the CCA mutant cells can be overcome by mutation at the relA locus. This material has been submitted to J. Mol. Biol.

Proposed Course of Project:

We will continue our efforts to identify functions of major outer membrane proteins.

A. Colicin and bacteriophage sensitivity. Some individual major outer

membrane proteins are involved in either (or both) colicin and bacteriophage sensitivity. We will try to define which proteins are involved in sensitivity to specific colicins and bacteriophages and to clarify the role of these proteins in penetration of the outer membrane barrier by these substances. The goal of these studies is an understanding of how individual proteins function in maintenance of the integrity of the outer membrane.

B. Permeability: It is likely that outer membrane proteins Ia and Ib have a role in the uptake of water soluble substances from the growth medium. We would like to use our mutants deficient in one or both of these proteins [as well as our mutant that may have an altered major outer membrane protein (see below)] to explore the role of these proteins in the permeability of the outer membrane.

C. Origin of a unique outer membrane protein:

We have isolated a mutant strain of E. coli that has a unique major outer membrane protein. We will determine the location of the mutation on the chromosome and examine the chemical and physiological properties of the unique protein per se.

1. Genetic studies: We will use standard genetic technique of conjugation and transduction to locate the mutation and determine whether it is dominant or recessive.

2. Chemical studies: We will solubilize the protein and purify it using techniques already developed for the purification of other major outer membrane proteins. Once purified, we will compare properties of this protein with those of other major outer membrane proteins, properties such as molecular weight, amino acid composition, N-terminal amino acid, cyanogen bromide and tryptic peptide analysis will be used.

3. Physiological studies: We will study the sensitivity of the mutant strain to various agents (colicins, bacteriophages, antibiotics, dyes, detergents, chelation agents). These agents probe the integrity of the outer membrane. In vitro studies using the purified protein will try to define any interaction of the protein with colicins or bacteriophages in the presence of other membrane components such as lipopolysaccharide or phospholipids.

The interest in the unique protein found in the outer membrane of this mutant is based upon an indication that this protein may represent an altered form (precursor?) of outer membrane proteins Ia and/or Ib. An understanding of this relationship may well lead to an understanding of how outer membrane proteins are synthesized and translocated specifically to the outer membrane.

Publications:

Foulds, J.: tolF locus in Escherichia coli: Chromosomal location and relationship to loci cmlB and tolD. J. Bacteriol. 126: 604-608, 1976.

Chai, T. and Foulds, J.: Escherichia coli K-12 tolF mutants: Alterations in protein composition of the outer membrane. J. Bacteriol. 130: 781-786, 1977.

Chai, T. and Foulds, J.: Purification of outer membrane protein A missing in Escherichia coli K-12 ompA mutants. Biochem. Biophys. Acta (in press).

Raetz, C. and Foulds, J.: Envelope composition and antibiotic hypersensitivity of Escherichia coli mutants defective in phosphatidylserine synthetase. J. Biol. Chem. (in press).

| SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space) | U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT** | PROJECT NUMBER Z01-AM-18000-12-LBM |
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PERIOD COVERED
July 1, 1976 to September 30, 1977

TITLE OF PROJECT (80 characters or less)
Hormone-Dependent Differentiation of Mammary Gland

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

| | | | |
|---|---|---|---|
| PI: | Dr. Yale J. Topper | Research Chemist, Chief, Section on Intermediary Metabolism | LBM NIAMDD |
| Other: | Dr. Colette S. Freeman | Senior Staff Fellow | LBM NIAMDD |
| | Dr. Tomasz Kuryl | Visiting Fellow | LBM NIAMDD |
| | Dr. Bruce D. Aidells | Staff Fellow | LBM NIAMDD |

COOPERATING UNITS (if any)

LAB/BRANCH
Laboratory of Biochemistry and Metabolism

SECTION
Section on Intermediary Metabolism

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20014

| TOTAL MANYEARS: | PROFESSIONAL: | OTHER: |
|---|---|---|
| 5.50 | 3.50 | 2.0 |

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Progesterone is strictly a growth-promoter for mammary epithelium. It is not required for its differentiation.

Modified insulin derived from insulin-Sepharose can stimulate ornithine decarboxylase activity in liver of insulin-resistant mice *in vivo*.



PHS-6040

Project Description:

Objectives - To study the molecular and cytological phenomena involved in development of the mammary gland. This includes attempts to understand the mechanism of action of insulin, prolactin, placental lactogen, glucocorticoids, estrogen and progesterone on this tissue. Also, to study the enhanced biological effects of an insulin-derivative derived from insulin-Sepharose.

Methods Employed -

A. In Vitro. Mammary gland explants, mammary epithelial cells or mammary cell nuclei are cultured in synthetic media. Synthesis of α-lactalbumin is followed by measuring the formation of lactose in tissue extracts. DNA and RNA synthesis are studied using conventional methods. DNA synthesis is inhibited by the addition of 1-β-D-arabinosylcytosine or fluorodeoxyuridine. Insulin-Sepharose, prolactin-Sepharose and placental lactogen-Sepharose are made by conventional methods. The intra-cellular accumulation of α-aminoisobutyric acid (AIB) is followed with the use of $^{14}$C-AIB or $^{3}$H-AIB. Glucose oxidation is assessed by measuring the conversion of $^{14}$C-glucose to $^{14}CO_2$.

B. In Vivo. Male mice, intact as well as hormone-ablated, are treated with various hormone combinations. Growth of the mammary epithelium is monitored in stained, whole-mount preparations. Differentiation is monitored by assaying for α-lactalbumin activity.

Major Findings -

A. The epithelium in the mammary gland of the adult male mouse resembles the anlage in the embryonic female. We have therefore used the male animal as a convenient source of embryonic-like mammary epithelium. It has been observed that appropriate hormone treatment in vivo can elicit growth and differentiation of the male epithelium equivalent to that which occurs in the pregnant female.

Using intact and hypophysectomized male mice, it has been determined that estrogen and prolactin are required for ductal growth. Alveolar growth requires progesterone, in addition.

Progesterone, although required for formation of alveoli, does not appear necessary for terminal differentiation of the ductal outgrowths resulting from stimulation by estrogen and prolactin. Thus, the ducts formed in castrated, adrenalectomized male mice can synthesize α-lactalbumin in vitro in the presence of insulin, glucocorticoid and prolactin. Progesterone, then, may serve solely as a growth-promoter for mammary epithelium, with no positive function in its differentiation.

B. There is at least one important qualitative difference between mammary epithelium from primiparous non-pregnant and mature virgin mice. Only

that from the virgin animals must traverse the cell-cycle in order to be able to synthesize milk-proteins in vitro in response to insulin, glucocorticoid and prolactin. Initial studies indicate that preventing such traversal has no detectable effect on ultrastructural development of the cells. But immunological analyses suggest that such blocked cells are not able to synthesize even the unphosphorylated polypeptide backbone of casein. Whether or not the corresponding m-RNA is transcribed under blocking conditions will be determined with the use of c-DNA.

C. We have reported that a modified form of insulin derived from insulin-Sepharose can elicit insulin-like effects in vitro from insulin-unresponsive tissues. Thus, whereas mammary epithelial cells from non-pregnant mice, and diaphragm from C57BL/ob ob mice do not respond to insulin, they do respond to the modified insulin. It was of interest to determine whether this difference in biological activity between the two forms of the hormone could also be demonstrated in vivo. Accordingly, liver ornithine decarboxylase (ODC) activity in the intact mouse has been studied. Insulin stimulates this activity 3-4 fold in the liver of lean C57BL mice, but has no stimulatory effect in C57BL/ob ob mice. The modified insulin does enhance ODC activity 3-4 fold in the liver of the obese, insulin-resistant mouse.

Proposed Course of Project -

A. Mammary alveolar formation under the influence of progesterone will be used as a model system for studying control of morphogenesis.

B. The observed qualitative difference between mammary epithelium from primiparous and virgin mice will be further studied in relation to risk of mammary cancer as a function of parity.

C. Additional biological effects of the modified insulin in intact insulin-resistant animals will be sought.

Significance to Biomedical Research and the Program of the Institute - Hormones are involved in normal growth and development and are implicated in many disease states. The mammary gland system offers an excellent opportunity to increase our understanding about the ways in which a number of different types of hormones interact with target cells.

Publications:

Topper, Y. J., Oka, T., Vonderhaar, B. K. and Wilchek, M.: An insulin-derivative with biological activity greater than that of native insulin. J. Cellular Physiology 89: 647-649, 1976.

Topper, Y. J.: Some hormonal determinants of normal mouse mammary epithelium. Excerpta Medica, in press.

Vonderhaar, B. K.: Studies on the mechanism by which thyroid hormones

enhance α-lactalbumin activity in explants from mouse mammary glands. Endocrinology 100: 1423-1431, 1977.

| SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space) | U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT | PROJECT NUMBER Z01 AM 18001-03 LBM |
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PERIOD COVERED
July 1, 1976 through September 30, 1977

TITLE OF PROJECT (80 characters or less)
Regulation of Glycolysis in Brain

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

| | | | |
|---|---|---|---|
| PI: | Dr. Frank Eisenberg, Jr. | Research Chemist | LBM NIAMDD |
| Other: | Dr. Arunendra L. Majumder | Visiting Associate | LBM NIAMDD |
| | Dr. Toshio Maeda | Visiting Fellow | LBM NIAMDD |

COOPERATING UNITS (if any)

LAB/BRANCH
Laboratory of Biochemistry and Metabolism

SECTION
Section on Intermediary Metabolism

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20014

| TOTAL MANYEARS: | PROFESSIONAL: | OTHER: |
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| 2.25 | 2.25 | 0 |

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Fructose 1,6-bisphosphatase, a key gluconeogenic enzyme, has been found in brain, a primarily glycolytic organ. The enzyme has been purified to 95% homogeneity and is essentially specific for fructose 1,6-bisphosphate. No other bisphosphate or monophosphate except sedoheptulose 1,7-bisphosphate (20% of fructose bisphosphate) is a substrate. Contrary to other mammalian fructose 1,6-bisphosphatases the brain enzyme requires no divalent cation and is not inhibited by 5'-AMP. The existence of this enzyme in brain completes a pathway that may provide for regulation of glycolysis in that organ.

Project Description:

Objective - Demonstration of fructose 1,6-bisphosphatase ($FruP_2ase$) in brain as predicted from a previous study of inhibition of brain glycolysis by phospholipid.

Methods - $FruP_2ase$ was purified from brain high speed supernatant by heat and acid treatment followed by chromatography on phosphocellulose and elution with fructose 1,6-bisphosphate ($FruP_2$). The activity was assayed as liberated inorganic phosphate. Purity was established by SDS polyacrylamide gel electrophoresis. Specific antibody was prepared and immunological properties were studied by Ouchterlony double diffusion and by precipitation of active enzyme with antibody.

Major Findings - Brain, primarily a glycolytic organ, has been shown to contain $FruP_2ase$, a key enzyme in gluconeogenesis. Previous attempts elsewhere to demonstrate the enzyme in brain have been unsuccessful. Although the amount of enzyme is comparable to that in liver the specific activity is only 1/200 of the liver enzyme, a fact which may account for the low gluconeogenic activity of brain. The enzyme has been purified 300-fold to 95% homogeneity and is virtually specific for $FruP_2$, no other bisphosphate or monophosphate serving as substrate except sedoheptulose 1,7-bisphosphate toward which the enzyme is 1/5 as active. $K_m$ for $FruP_2 = 1.32 \times 10^{-4}$ M. In contrast to other mammalian $FruP_2ases$, brain $FruP_2ase$ has no metal requirement and is not inhibited by 5'-AMP or EDTA; it is maximally active above pH 7.6. The enzyme cross reacts with its own antibody but not with antibody to the liver enzyme, showing differences between the two enzymes although they both have the same subunit molecular weight of 40,000. The enzyme is quantitatively precipitated by its antibody and the precipitated complex is enzymatically active. The enzyme is not stimulated by phospholipid and therefore cannot account for the inhibition of glycolysis in brain by phospholipid reported previously.

Significance to Biomedical Research and the Program of the Institute - Regulation of the flow of metabolites is an important aspect of cellular physiology and its derangement can lead to pathological states. Since glucose 6-phosphate is the primary fuel of the brain its proper partition among the various pathways of utilization is important to the function of that organ. With the demonstration of $FruP_2ase$ in brain glycolysis may be under regulation through reversal, i.e. gluconeogenesis.

Proposed Course of Project - Since the inhibitory effect of phospholipid on glycolysis has been localized at the interconversion of the fructose phosphates, but is not attributable to $FruP_2ase$, the responsible enzyme must be phosphofructokinase (PFK). Although PFK in other tissues is known to be inhibited by phospholipid, the effect has not been examined with purified brain PFK. That study will be undertaken as well as attempts to demonstrate a physiological role for phospholipid in brain glycolysis.

Publications:

Majumder, A. L. and Eisenberg, F., Jr.: Inhibition of glycolysis in brain by a phospholipid effect on the interconversion of fructo — phosphates. A possible regulatory control on utilization of glucose 6-phosphate. J. Biol. Chem. 251: 7149-7156, 1976.

Majumder, A. L. and Eisenberg, F., Jr.: The unequivocal demonstratio of fructose 1,6-bisphosphatase in mammalian brain. Proc. Nat. Acad. Sci. USA, in press.

| SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space) | U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT** | PROJECT NUMBER Z01 AM 18002-04 LBM |
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PERIOD COVERED
July 1, 1976 to September 30, 1977

TITLE OF PROJECT (80 characters or less)
Investigations on the Biochemical Abnormality Underlying Human Cystinosis

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: Dr. Frank Tietze Research Chemist LBM NIAMDD

COOPERATING UNITS (if any)
Dr. Joseph D. Schulman, Dr. E. Jean Butler: Section on Human Biochemical and Developmental Genetics, NPMB, NICHHD.

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SECTION
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| TOTAL MANYEARS: | PROFESSIONAL: | OTHER: |
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| 0.75 | 0.75 | 0 |

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☐ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Cultured skin fibroblasts from patients with I-cell disease, an inherited lysosomal disorder characterized by multiple acid hydrolase deficiency, were assayed with respect to their intracellular content of the amino acid cystine. Seven of these lines were found to contain abnormally elevated levels of this amino acid in a manner reminiscent of the unrelated lysosomal storage disorder cystinosis. Several lines from patients with mucolipidosis III, a biochemically similar but clinically less severe variant of I-cell disease, were normal with respect to their intracellular cystine levels. The finding of supra-normal levels of cystine in I-cell fibroblasts may be relevant to an understanding of the pathogenesis of human cystinosis.



PHS-6040
(Rev. 10-76)

Project Description:

Objectives - To define the biochemical basis of cystinosis, a rare and often fatal inherited disease of children and young adults characterized by the abnormal accumulation of the amino acid cystine within the lysosomes of certain cells of the body.

Methods - Cultured skin fibroblasts from normal individuals and from patients with cystinosis or other lysosomal storage diseases of particular interest are washed free of external medium, collected by centrifugation following trypsin treatment, and disrupted by addition of sulfosalicylic acid. The supernatant protein-free fluid is then assayed for cystine by one of several analytical methods.

Major findings - Ten different fibroblast cell lines from patients with I-cell disease, an inherited lysosomal storage disorder unrelated to cystinosis, have been examined with respect to their intracellular cystine content. Of these lines, seven were found to have distinctly supra-normal levels (> 2 nanomoles half-cystine per mg protein) of this amino acid, one was marginally elevated, and two were normal (i.e., ca. 0.5 nanomoles/mg). Cystine levels from three cell lines from the biochemically similar, but clinically less severe variant of I-cell disease, viz., mucolipidosis III, were within normal limits, as were the levels in lines from patients with three different forms of mucopolysaccharide storage diseases.

Significance to Biomedical Research and the Program of the Institute - Human cystinosis is an interesting but puzzling example of a lysosomal storage disease in that the storage substance is a simple amino acid, the catabolism of which is not known to involve the participation of a lysosomal acid hydrolase. Since cultured I-cells are known to be multiply-deficient with respect to an array of acid hydrolase activities, the finding that some of these cells exhibit a pseudo-cystinotic condition is compatible with a lysosomal defect in the pathogenesis of cystinosis.

Proposed Course of Project - Further characterization of the pseudo-cystinotic character of I-cell fibroblasts will be undertaken. It will be of interest to determine whether treatment of these cells with agents such as cysteamine and ascorbic acid, which are known to bring about significant reduction in cystine levels in cultured fibroblasts from cystinotic individuals, will produce similar effects in cultured I-cells.

Publications:

Butler, J., Tietze, F., Pellefigue, F., Spielberg, S. and Schulman, J. D.: Depletion of cystine in cystinotic fibroblasts by drugs enclosed in liposomes. Pediatric Research, in press.

| SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space) | U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT** | PROJECT NUMBER Z01-AM-18003-04 LBM |
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PERIOD COVERED
July 1, 1976 through September 30, 1977

TITLE OF PROJECT (80 characters or less)

Spermidine and the development of mammary gland.

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

| | | | |
|---|---|---|---|
| PI: | Dr. Takami Oka | Research Chemist | LBM NIAMDD |
| Other: | Dr. Kazutaka Kano | Visiting Fellow | LBM NIAMDD |
| | Dr. Tadashi Sakai | Visiting Associate | LBM NIAMDD |
| | Mr. John W. Perry | Research Assistant | LBM NIAMDD |

COOPERATING UNITS (if any)

None

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INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20014

| TOTAL MANYEARS: | PROFESSIONAL: | OTHER: |
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| 4.0 | 3.0 | 1.0 |

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Our previous studies indicated that the polyamine spermidine plays an important regulatory role in the development of mammary epithelium. To elucidate the control mechanism for spermidine biosynthesis, we have purified S-adenosyl-L-methionine decarboxylase, a rate-limiting enzyme, to apparent homogeneity by various chromatographic procedures, and studied various properties of the enzyme. The enzyme activity is markedly stimulated by putrescine (Ka=0.5 μM) which lowers the apparent Km for the substrate and also prevents inactivation of the enzyme. In contrast, spermine inhibits the enzyme activity by reducing Vmax of the enzyme. Since putrescine and spermine are intimately related to the decarboxylase reaction in the polyamine biosynthetic pathway, they may act as regulatory factors in cellular control of polyamine biosynthesis via their action on the decarboxylase.

To pursue further the elucidation of the mechanism of action of spermidine which mimicks the action of glucocorticoid on milk-protein synthesis in cultured mammary cells, we have examined the effect of glucocorticoid on the transcription of casein mRNA. The results show that the steroid, in the presence of insulin, enhances the accumulation of the mRNA, which remains untranslated in the absence of prolactin.

Project Description:

The polyamine spermidine is present ubiquitously in living cells, and is currently considered to play an important regulatory role in the process of growth and development of mammalian cells.

In mammary epithelium, the concentration of spermidine increases markedly during lactation. A similar increase has been found during the hormonal induction of lactogenesis in vitro, which is effected by cultivation of mouse mammary explants with insulin, glucocorticoid and prolactin. Previous studies on the role of spermidine with this in vitro system have presented several lines of evidence indicating that the polyamine may serve a vital function in milk-protein synthesis, possibly by mediating the effect of glucocorticoid.

Based on these previous findings, we have continued a series of studies to elucidate the mechanism of hormonal control of spermidine formation during mammary development in vitro. In addition, we have extended our studies to dissect the mechanisms of action of spermidine at sub-cellular and molecular levels.

The experimental procedures employed in this investigation include the following: organ culture of mouse mammary gland; preparation of mammary epithelium free of fat cells; isolation and quantification of the polyamines; enzyme assays for ornithine decarboxylase, S-adenosyl-L-methionine decarboxylase, arginase, spermidine synthase, and α-lactalbumin; a number of column chromatography techniques, such as DEAE, Sephadex, hydroxyapatite, and affinity chromatography; isolation of polysomes and various species of RNAs by sucrose density gradient centrifugation; oligo dT-cellulose and poly U-Sepharose chromatography; characterization of macromolecules by SDS-gel electrophoresis, and development of casein antibody and a cell-free polypeptide synthesizing system.

Our studies on S-adenosyl-L-methionine decarboxylase and spermidine synthase, the two enzymes which are involved in the final step of spermidine biosynthesis, have shown that the activities of these two enzymes are stimulated by the actions of insulin and glucocorticoid. The decarboxylase has been purified to apparent homogeneity and its properties have been extensively studied. Using the pure form of the decarboxylase, we are developing the antibody for the enzyme, which is to be used for assessing the mechanism of the hormonal regulation of the decarboxylase.

To elucidate the mechanisms of action of spermidine in milk-protein synthesis at the molecular level, we have focused on the transcriptional and the translational control of casein mRNA. To this end, we examined the effect of glucocorticoid on the metabolism of casein mRNA in cultured mammary explants. These studies showed that glucocorticoid, in the presence of insulin, facilitates the accumulation of the mRNA, which remained untranslated without further exposure of mammary cells to prolactin. The results suggest

the "two-step" regulation of milk-protein synthesis, one at the transcriptional and the other at the translational level. Glucocorticoid or spermidine appears to prolong the half-life of the untranslated casein mRNA.

We intend to pursue further the elucidation of the regulatory mechanism for the biosynthesis of spermidine using antibody for S-adenosyl-L-methionine decarboxylase, and to attempt to dissect the mechanism of action of spermidine, insulin, glucocorticoid and prolactin at molecular levels. In addition, the possible role of spermidine in mammary cell proliferation will be studied.

An understanding of the action of the polyamines may serve to reveal the complex regulatory mechanisms of tissue growth and development. Such knowledge would certainly be useful for diagnosis and treatment of various pathophysiological disorders, including cancer and other metabolic diseases.

Publications:

Kano, K. and Oka, T.: Polyamine transport and metabolism in mouse mammary gland. General properties and hormonal regulation. J. Biol. Chem. 251: 2795-2800, 1976.

Oka, T., Kano, K. and Perry, J. W.: Spermidine and the action of glucocorticoid during the development of mammary gland. Excerpta Medica, in press.

Oka, T., Kano, K., and Perry, J. W.: Studies on S-adenosyl-L-methionine decarboxylase of mouse mammary gland. Proceedings of the Pacific Northwest Conference on Polyamines, Raven Press, in press.

| SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space) | U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT** | PROJECT NUMBER Z01 AM 18004-04 LBM |
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PERIOD COVERED
July 1, 1976 to September 30, 1977

TITLE OF PROJECT (80 characters or less)
Biochemical Lesions in Genetic Mucopolysaccharidoses

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: Dr. Irwin G. Leder Research Chemist LBM NIAMDD

COOPERATING UNITS (if any)
Section on Human Biochemical Genetics, ARB, NIAMDD

LAB/BRANCH
Laboratory of Biochemistry and Metabolism

SECTION
Section on Intermediary Metabolism

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20014

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Two mucopolysaccharidoses, the Hunter syndrome and the Hurler syndrome, are lysosomal disorders due to the absence, respectively, of iduronate sulfatase and iduronidase. Specific substrates have been prepared and radiological assay procedures for the purification and study of these enzymes have been developed. These procedures have been applied to the diagnosis of the corresponding genetic disease and to the detection of the heterozygous state.



PHS-6040
(Rev. 10-76)

Project Description:

Objectives - To study the hereditary biochemical defects of lysosome dysfunction which are responsible for a group of interrelated mucopolysaccharidoses and to apply the results of such studies to clinical diagnosis and therapy.

Methods Employed - Substrates for iduronidase, iduronate sulfatase and glucosamine-N-sulfatase are prepared by controlled nitrous acid degradation of heparin and by synthesis. Appropriate structural units are modified by desulfation, sulfation with [$^{35}$S]sulfur trioxide complexes and by tritiation with borotritide. These substrates are used to assay enzymes in preparative and clinical diagnostic procedures and to prepare derivatives for affinity chromatography.

Major Findings -

1. The isolation and tritiation of the basic disulfated disaccharide substrate have been significantly improved. Previously from 5-10% of the isolated disaccharide was resistant to borohydride reduction. Modification of the procedure results in complete reduction of the anhydromannose moiety. Samples of the substrate have been made available to laboratories in the United States and abroad.

2. Iduronate sulfatase substrate of very high specific activity has been prepared and applied to the detection of the heterozygous state by assaying the enzymatic activity in hair root follicles.

Significance to Biomedical Research and the Program of the Institute - The development of techniques for studying the genetic mucopolysaccharidoses is of medical significance in terms of diagnosis, genetic counseling and possible treatment. The availability of specific substrates and of sensitive biochemical assays has simplified diagnostic procedures and has broadened the scope of genetic counseling. The purification of the enzymes associated with the mucopolysaccharidoses from human sources and the clarification of the "high uptake" process may lead to clinical trials in enzyme replacement therapy.

Proposed Course of Project -

1. The Sanfilippo-A disease is caused by a deficiency of a glucosaminide N-sulfatase. Biochemical diagnosis of the disease and study of the enzymes have been difficult because an appropriate low molecular weight substrate is not available. Two approaches to providing such a substrate will be pursued; the synthesis of simple N-sulfated glucosaminides and the isolation of tetrasaccharide units from heparin by partial degration with nitrous acid. Preliminary tests with N-sulfated methyl glucosaminide have been inconclusive because of the high background of amino groups present in enzyme preparations. The corresponding [$^{35}$S] derivative will be prepared

to overcome this difficulty.

2. A Sepharose disaccharide disulfate derivative, which has been shown to bind iduronate sulfatase, will be tested in affinity chromatography studies of enzyme purification.

Publications:

DiNatale, P., Leder, I. G. and Neufeld, E. F.: A radioactive substrate and assay for α-L-iduronidase. Clinica Chimica Acta, in press.

| SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space) | U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT | PROJECT NUMBER Z01 AM 18005-04 LBM |
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PERIOD COVERED
July 1, 1976 to September 30, 1977

TITLE OF PROJECT (80 characters or less)

Differentiation of Lymphoid Cells

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

| | | | |
|---|---|---|---|
| PI: | Dr. Milton Kern | Research Chemist | LBM NIAMDD |
| Other: | Dr. Stephen H. Gregory | Staff Fellow | LBM NIAMDD |

COOPERATING UNITS (if any)
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Bethesda, Md. 20014

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Laboratory of Biochemistry and Metabolism

SECTION
Section on Intermediary Metabolism

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20014

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

A procedure has been devised for the iodination of lipid A. The resultant product, $^{125}$I-lipid A, retains its mitogenic activity and exhibits properties like that of untreated lipid A. In accord with what would be expected from the known mitogenic specificity of both lipid A and its parent compound lipopolysaccharide, $^{125}$I-lipid A binds to B cells but not T cells. This finding suggests that the cellular specificity resides in an early event in mitogenesis.

The mitogenic and antimitogenic activity of certain nucleosides and nucleotides on spleen and thymus cells were demonstrated. There was no consistent correlation between the effect of these agents on enhancing the cellular content of cyclic AMP or cyclic GMP and on enhancing or inhibiting DNA synthesis. Inhibition of DNA synthesis by certain nucleotides was not reversible but it did not result in inhibition of protein synthesis.

Enzymes predominantly found in the plasma membrane are not uniformly distributed among cells of the lymphoid series. 5'-Nucleotidase and non-specific diesterase are readily observed in spleen but barely detectable in thymocytes of the same animals. These activities in spleen are not associated with the bulk of either T cells or B cells.

Project Description:

Objectives - The purpose of this project is to study the differentiation of the cells involved in the immune response with regard to cell types, cell interactions and control mechanisms. During this report period the experimental work emphasized was as follows: (A) analysis of the cellular specificity of binding of the mitogen lipid A; (B) analysis of the mitogenic and anti-mitogenic activity of nucleosides and nucleotides, and (C) analysis of the distribution of enzymes that are primarily associated with plasma membrane among the different cells involved in the immune response.

Methods Employed - Rabbit lymphoid cells were incubated in tissue culture medium for 72 hours at 37° C. The cells were then harvested, washed in leucine-free medium and incubated in such medium supplemented with [3H]-leucine for 90 minutes. Immunoglobulin production was judged from the quantity of [3H]-immunoglobulins secreted into the extracellular fluid. [3H]-Immunoglobulins were assessed by coprecipitation in the presence of carrier rabbit immunoglobulin and antisera specific for rabbit immunoglobulin. DNA synthesis was analyzed by following the incorporation of [3H]-thymidine into DNA.

Major Findings - Bacterial lipopolysaccharide (LPS) is a selective mitogenic agent in that it induces enhanced DNA synthesis of bone marrow-derived cells (B cells) but not thymus-derived cells (T cells). Moreover, the lipid A moiety of LPS accounts for the mitogenic effects of LPS.

In order to study the means by which lipid A activates B cells but not T cells, it was first necessary to devise a procedure for labeling lipid A so that binding studies could be performed. An iodination technique was devised which provided a product having the appropriate chemical characteristics as well as the relative sensitivity that might be expected of an $^{125}I$-labeled indicator molecule. Such iodinated lipid A possesses the full mitogenic activity of untreated lipid A. Comparison of the $^{125}I$-lipid A binding activity of splenocytes and thymocytes from the same rabbit revealed that the extent of labeling of splenocytes was 10-20 times greater than that observed with an equivalent number of thymocytes. A similar preferential binding was detected in comparing cells in mouse and rat. Non-adeherent spleen cell populations were essentially unaltered with regard to binding when compared to the original population. In addition, spleen cell populations enriched for T cells exhibited a marked loss of specific binding activity. On the other hand, spleen cell populations enriched for B cells exhibited the expected binding. The difference in binding behavior of B cell- and T cell-enriched populations was confirmed using three independent techniques to separate B cells and T cells. These findings are consistent with the mitogenic specificity of lipid A toward B cells rather than T cells and suggests that the observed cellular specificity resides in an early event in mitogenesis, i.e., binding of the mitogen.

At a concentration of $10^{-3}$ M a variety of nucleosides and nucleotides was found to have a pronounced effect on the induction of DNA synthesis by spleen and thymus cells incubated in tissue culture medium containing fetal

calf serum. In some cases nucleosides and nucleotides were inhibitory and in others stimulatory. Spleen cells and thymus cells showed several differences in their behavior. For example, murine spleen cells cultured with GMP-5' exhibited enhanced DNA synthesis (about 3-fold), while the presence of AMP-5' during cell culture markedly inhibited DNA synthesis (>90%). On the other hand, murine thymus cells showed enhanced DNA synthesis in the presence of both AMP-5' and GMP-5' (5-fold). This disparity in behavior extends further in that DNA synthesis by rabbit thymus cells was inhibited by AMP-5', in contrast to findings with murine thymocytes. However, rabbit thymocytes yielded an enhanced thymidine incorporation in the presence of GMP-5'.

Although both AMP-5' and GMP-5' induce an increase in the intracellular levels of cyclic nucleotides, there was no consistent correlation in regard to the effects of AMP-5' and GMP-5' on DNA synthesis. Inhibition of DNA synthesis by AMP-5' appears to be an irreversible process and is selective in the sense that protein synthesis was essentially unaffected.

The fact that some nucleotides can be mitogenic as well as antimitogenic along with the commonly-held notion that phosphorous-containing compounds do not pass the cell membrane barrier, suggested the need for examining the distribution of plasma membrane-associated enzymes among the cells involved in the immune response as a prelude to understanding the possible function of ectoenzymes in the induction of DNA synthesis and immunoglobulin production. It has been observed that rabbit spleen lymphocytes possess as much as 300 times the level of 5'-nucleotidase as found in an equivalent number of thymus cells. The enzyme activity in spleen populations is not principally associated with either T cells or B cells. Moreover, 5'-nucleotidase activity is not observed in bone marrow, appendix or buffy coat cell populations.

Preliminary studies with other ectoenzymes reveal that sialyl-transferase and nucleotide pyrophosphatase activity levels are essentially the same in spleen and thymus and that non-specific diesterase shows the unequal distribution observed with 5'-nucleotidase.

Significance to Biomedical Research and the Program of the Institute - A knowledge of the control mechanisms involved in the induction of immunoglobulin production will have vital importance not only in understanding how the organism reacts to foreign antigens, but also may be expected to affect significantly the approach used in regard to a number of immune diseases, e.g., immunodeficiency diseases, allergic disease, autoimmune disease and neoplastic disease of lymphoid cells.

Proposed course of project - Efforts will be directed towards studying the mechanism by which certain nucleosides and nucleotides affect the activation of lymphoid cells. The role of ectoenzymes in the immune response will be studied by preparing monospecific antisera against selected surface membrane enzymes and using immunofluorescence microscopy in order to determine the precise cells bearing enzyme. In addition, the effect of such antisera on the immune response will also be examined.

Publications:

Kern, M.: Surface immunoglobulins: Characteristics, mobility and role in immune phenomena. In Jamieson, G. A. and Greenwalt, P. J. (Eds.): Mammalian Cell Membranes. London, Butterworth & Co., 1977, pp. 98-113.

Shinohara, N. and Kern, M.: Differentiation of lymphoid cells: B cell as a direct target and T cell as a regulator in lipopolysaccharide enhanced induction of immunoglobulin production. J. Immunol. 116: 1607-1612, 1976.

Shinohara, N. and Kern, M.: Differentiation of lymphoid cells: The non-mitogenic induction of immunoglobulin production by thymus cell extract and thymus cell culture filtrate. Immunology 31: 433-441, 1976.

Zimmerman, D. H. and Kern, M.: A simple procedure for labeling the lipid A moiety of lipopolysaccharide with $^{125}I$. Anal. Biochem. 71: 340-345, 1977.

| SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space) | U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT** | PROJECT NUMBER Z01 AM 18006-06 LBM |
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PERIOD COVERED
July 1, 1976 to September 30, 1977

TITLE OF PROJECT (80 characters or less)
Particulate Enzymes of Carbohydrate Metabolism

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

| | | | |
|---|---|---|---|
| PI: | Dr. Marjorie R. Stetten | Research Chemist | LBM NIAMDD |
| Other: | Dr. Paul K. Goldsmith | Research Assistant | LBM NIAMDD |

COOPERATING UNITS (if any)

LAB/BRANCH
Laboratory of Biochemistry and Metabolism

SECTION
Section on Intermediary Metabolism

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The properties of a number of enzymes occurring in the endoplasmic reticular membranes of mammalian liver and kidney are being studied and compared with corresponding enzymes of marine invertebrates. Levels of the activities of glucose-6-phosphatase, $PP_i$-glucose phosphotransferase, NADPH-cytochrome c reductase, nucleoside diphosphatase and S-palmitoyl-coenzyme A:*sn*-glycerol-3-phosphate acyltransferase, studied as a function of the age of the animals, have revealed differing patterns of change during the neonatal and weaning periods, indicating that enzyme development is more closely related to the changing metabolic needs of the animal than to membrane proliferation. Differing latency properties of some of these enzymes are being studied as clues to enzyme location on or within the structure of the membrane.



PHS-6040
(Rev. 10-76)

Project Description:

The objective of the project has been to study the occurrence, properties and functions of some of the membrane-bound enzymes of liver and kidney which catalyze the synthesis, hydrolysis and transport of a variety of phosphorylated sugars and sugar alcohols. The quantitative levels of these enzymes have been found to be particularly susceptible in vivo to fasting and to a number of endocrine changes, such as result from diabetes, adrenalectomy, adrenocorticosteroid and insulin administration.

We have continued our studies of postnatal developmental changes in the activities of several of the key enzymes occurring in endoplasmic reticular membranes of liver and kidney. Glucose-6-phosphatase and its associated $PP_i$-glucose phosphotransferase activity, NADPH-cytochrome c reductase, nucleoside diphosphatase and S-palmitoyl-coenzyme A:sn-glycerol-3-phosphate acyltransferase were studied in rats as a function of the age of the animals. These enzymes were selected because of their location within the membrane and their possible role in the synthesis of membrane components or importance to the development of metabolism. Since glucose-6-phosphatase and nucleoside diphosphatase have latent activities which can be expressed by agents which disrupt membranes, we have also studied age-related changes in the latencies of these activities.

Our results for rat liver indicate the existence of several distinct patterns of enzyme development. Glucose-6-phosphatase of liver increased rapidly after birth to a level several-fold higher than that in adult liver and declined with age, while nucleoside diphosphatase gradually increased to reach adult levels. NADPH-cytochrome c reductase and S-palmitoyl-coenzyme A: sn-glycerol-3-phosphate acyltransferase underwent essentially parallel changes in activity with age, increasing and decreasing during the neonatal period before increasing again to adult levels during the weaning period.

The development of glucose-6-phosphatase in the kidney was found to be strikingly different from that in the liver. Both the hydrolase and the phosphotransferase activities, which were very low at birth, underwent a small increase and decrease in the neonatal kidney followed by a gradual increase in activity. The same activities in liver fell rapidly from the very high levels reached a few days after birth until the kidney and liver enzymes were at approximately the same levels in adult animals. In contrast, the other three enzymes underwent approximately parallel age-related changes in kidney and liver with the absolute levels remaining lower in kidney.

During the neonatal period all the enzymes studied had higher activities in rough than in smooth liver microsomes. Glucose-6-phosphatase differed from the other enzymes in exhibiting non-parallel changes with age in rough and smooth membranes. Glucose-6-phosphatase activity of rough membranes increased greatly during the first few days of life, while the activity in smooth membranes showed no such neonatal overshoot. Our results indicate that there is no general pattern of acquisition for membrane-bound enzymes

of proliferating ER membranes. Clearly the development of these enzymes is more closely related to the changing metabolic requirements of the animal than to membrane proliferation.

The degree of latency of glucose-6-phosphate phosphohydrolase has been found to be very much greater in the neonatal than in the adult liver. In contrast, the transferase function of the enzyme retains a relatively high degree of latency throughout life. In this regard it resembles nucleoside diphosphatase which is known to have a luminal location on the microsomal membrane. Since the location of the active sites of glucose-6-phosphatase, a phospholipid-requiring enzyme which is probably an integral part of the endoplasmic reticular membrane, has been the subject of a number of conflicting hypotheses, we propose to extend our latency studies to include the relative effectiveness of proteolytic enzymes and the effects of varying concentrations of detergents which are known to change membrane permeability. An attempt will be made to determine if the observed properties can be explained by a transmembrane localization of the enzyme molecule or if the existence of a specific substrate transport mechanism must be evoked.

Related experiments, carried out in part at the Marine Biological Laboratory at Woods Hole, Massachusetts, were designed to study aspects of the evolutionary development of sugar transport and utilization. The Chelicerate, Limulus polyphemus, was found to have an enzyme in its hepatopancreas and coxal glands with properties similar to those of the liver and kidney glucose-6-phosphatase of vertebrates, while most invertebrates lack this enzyme and Crustaceans have a similar enzyme with differing properties. These findings, together with the known utilization by insects of the disaccharide trehalose for storage and transport of energy, have led us to propose that it was at the evolutionary level of the Arthropods that a variety of transport compounds was tried and that the specific particulate enzyme glucose-6-phosphatase occurs only when free glucose is an important means of energy transport. This problem is being extended to include a more detailed study of the nature of the glucose-6-phosphatase-like enzyme of Crustacean hepatopancreas and a phylogenetic survey of the occurrence and properties of the hexokinases of invertebrates.

Publications:

Stetten, M. R. and Goldsmith, P. K.: A Limulus glucose-6-phosphatase with phosphotransferase activity characteristic of vertebrate liver microsomes. Its possible evolutionary significance. Biochim. Biophys. Acta 444: 835-852, 1976.

| SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space) | U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT** | PROJECT NUMBER Z01 AM 18007-01 LBM |
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PERIOD COVERED
July 1, 1976 to September 30, 1977

TITLE OF PROJECT (80 characters or less)
Possible Functional Relationship of Glutathione and Polyamines in Mammalian Cells

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT


PI: Dr. Frank Tietze Research Chemist LBM NIAMDD

PI: Dr. Takami Oka Research Chemist LBM NIAMDD


COOPERATING UNITS (if any)

LAB/BRANCH
Laboratory of Biochemistry and Metabolism

SECTION
Section on Intermediary Metabolism

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20014

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)


During the early growth phase of cultured human skin fibroblasts, the uptake of [$^3$H]spermidine and the intracellular content of total glutathione increase together in a parallel manner to maximum values in the first 24-48 hours, then decline together during the proliferative phase, ultimately reaching low steady-state values at confluency. The apparent correlation in the behavior of these two parameters may be an indication of a mutually functional role for these two substances in cellular growth or metabolism.





PHS-6040
(Rev. 10-76)

Project Description:

Objectives - To assess the possible functional interrelationship of glutathione (GSH) and polyamines during the growth cycle of normal and mutant human cells in culture.

Methods - Cultured human skin fibroblasts from normal subjects and from patients with inherited defects in glutathione metabolism are subcultured in small plastic dishes and allowed to proliferate. At intervals of time, including zero time, samples are withdrawn and incubated for 90-120 minutes with fresh medium containing labeled [$^3$H]spermidine. Following the incubation, the cells are brought into suspension by treatment with trypsin, counted, washed free of external label by centrifugation and finally disrupted with dilute acid. One portion of the resultant lysate is counted by liquid scintillation while a second portion is assayed for total glutathione by a sensitive spectrophotometric method.

Major findings - During the first 24-48 hours following subculture of normal human fibroblasts the uptake of spermidine and the intracellular glutathione levels increase rapidly and in a strikingly parallel manner; these quantities decline during the subsequent logarithmic growth period, again in an apparently coordinated manner and ultimately attain low steady state levels at confluency. Similar parallel changes were observed in mutant human cell lines deficient in one of the several enzymes involved in GSH metabolism, although levels of spermidine uptake, GSH content, and growth rate were considerably altered in comparison with normal cells.

Significance to Biomedical Research and the Program of the Institute - Both polyamines and glutathione are universally distributed in all living cells, where they are believed to play essential roles in cellular growth and metabolism. Although GSH and spermidine have been found in chemically linked form in E. coli, their functional association in mammalian cells has not been established. The correlation between spermidine uptake and glutathione content during the growth cycle of cultured human fibroblasts observed in this investigation suggests the possibility of such an association in mammalian cells and may, in addition, be relevant to the biochemical consequences of some inherited abnormalities involving glutathione known to occur in human subjects.

Proposed Course of Project - Further experiments designed to establish more firmly a functional relationship between polyamine uptake and GSH levels will be conducted. Particular emphasis will be placed on the reciprocal effects of inhibitors of GSH metabolism and of polyamine biosynthesis as a means of establishing this relationship.

Publications:

None.

ANNUAL REPORT OF THE LABORATORY OF CHEMISTRY

NATIONAL INSTITUTE OF ARTHRITIS, METABOLISM AND DIGESTIVE DISEASES

SECTION ON BIOCHEMICAL MECHANISMS

Chemistry of Imidazoles

The unusual physical and chemical properties of substituted imidazoles, which are so intimately related to their biological properties, have been the subject of further investigation. We have demonstrated that the exceptionally low basicities of fluoroimidazoles are not anomalous, since the $pK_1$ values of ten fluoroimidazoles can be included with seventy other mono- and polysubstituted imidazoles in a correlation with aromatic ortho substituent constants [L.A. Cohen and S. Takahashi, JACS, 95, 443 (1973)]. Values of $pK_2$ also correlate well with this scale. Thus, the acidic or basic pK of any new imidazole can now be predicted with high accuracy. The generally accepted correlations with meta sigma constants must now be considered invalid.

The anomalous nmr spectra of haloimidazoles have also been explained. For protons attached to $sp^3$ carbon, chemical shifts are determined by the electronegativities of chain substituents; for protons on aromatic and heteroaromatic carbon, however, only the pi-electron density is significant and $\delta$ values for imidazole protons have been correlated successfully with the resonance component of aromatic $\sigma$ constants [L. A. Cohen and W. M. Jones, JACS, 85, 3402 (1963)]. Efforts to extend these correlations to $^{13}C$ nmr values are in progress.

Studies on acid-catalyzed hydrogen isotope exchange in fluoroimidazoles led to the prediction that nitration of these compounds should occur under very mild conditions. Indeed, the prediction has been verified and intermediates for the synthesis of 2,4-difluoroimidazoles have become available for the first time. Development of the triphenylmethyl (trityl) blocking group for imidazole nitrogen represents a major advance in synthetic imidazole chemistry, providing the first general route to 2-substituted imidazoles (including histamines and histidines) and to difficultly accessible 4-substituted imidazoles. The procedure involves generation of a carbanion at C-2 of the N-tritylimidazole with butyl lithium, reaction with an electrophilic reagent, and removal of the blocking group in dilute acetic acid. A novel route to 2-trifluoromethylhistidine has been discovered, based on the Bamberger cleavage of histidine with benzoyl chloride and reaction of the product with trifluoroacetic anhydride. This analog of histidine will be very useful in evaluating the significance of an acidic ring NH on the biological properties of the amino acid. Additional members of the imidazole trimer series have been synthesized, including tristidine (a cyclic trimer of histidine); these compounds are being evaluated as mutagenic agents. Photochemical fluorination, developed originally to make fluoroimidazoles, has been extended to other ring systems: e.g., 2-fluoromorphine has been synthesized from 2-nitromorphine. [L.A. Cohen, K.L. Kirk, Y. Takeuchi, S. Woods, H. Kimoto, P. Cohen and R. Jerussi (FDS)].

## Biochemistry and Pharmacology of 2-Fluoro-L-histidine

Administration of a nonlethal dose of 2-fluorohistidine (2-FHis) to mice produces a time- and dose-dependent leucopenia, temporary regression of the spleen, thymus, and lymph nodes, and hypocellularity of bone marrow. Administration of the histidine analog (150-250 mg/kilo) to mice infected with P-388 leukemia virus prevents the normally massive increase in leukocyte levels and increases survival time up to 50%. Ultimate death of the mice may be due to causes other than the orginal leukemia, a phenomenon now under investigation. We had shown previously that 2-FHis is rapidly incorporated into new protein (in place of histidine) in all murine organs, and it was assumed initially that the effect on leukocyte levels and on leukemia was a result of the biosynthesis of false protein. However, simultaneous administration of histidine or inhibitors of protein synthesis failed to counteract the effects of 2-FHis, these results suggesting that other inhibitory pathways may be involved. In an earlier study [C. B. Klee, L. E. La John, K. L. Kirk, and L. A. Cohen, BBRC, 75, 674 (1977)], 2-fluorourocanic acid, the first product of the catabolism of 2-FHis, was found to be a potent inhibitor of urocanase, the second enzyme in the pathway for histidine degradation. Since it is known that C-2 of the imidazole ring of histidine can be ultimately incorporated into purine bases via N-formyltetrahydrofolate, 2-fluorourocanic acid may serve to retard the utilization of histidine as a one-carbon source for purine biosynthesis; 2-FHis would then function as an in vivo source of 2-fluorourocanic acid. Most antileukemic agents in current use also retard purine biosynthesis by inhibition of dihydrofolate reductase and fluorourocanic acid may be retarding an earlier step in the same biosynthetic sequence. This hypothesis is strengthened by the observation that 2-FHis retards the overall growth of E. coli, but does not block the division of preexisting multinuclear cells. Whether histidine serves as an obligatory or secondary one-carbon donor has never been established; 2-FHis and 2-fluorourocanic acid, the first useful inhibitors of histidine catabolism in mammalian systems, may help settle the question. [L. A. Cohen, K. L. Kirk, C. R. Creveling (NIAMDD), and B. Highman (National Center for Toxicology, Jefferson, Arkansas).

Studies are continuing on the incorporation of 2-FHis into bacterial and animal protein, with the particular goal of isolating active or inactive enzymes containing fluorine; efforts to obtain biosynthetic fluorohemoglobin are also in progress [L. A. Cohen, K. L. Kirk, C. R. Creveling (NIAMDD)]

## Halogenated Biogenic Amines in Biochemistry and Pharmacology

5-Hydroxytryptamine (5-HT) is a substrate for monoamine oxidase A (MAO-A), but not for MAO-B. In contrast, 6-fluoro-5-HT and 4,6-difluoro-5-HT are substrates for both the A and B enzymes. Tyramine is a substrate for both MAO-A and MAO-B, but becomes a substrate only for the B enzyme following ring fluorination. It has been proposed that MAO-B requires more lipophilic substrates than does MAO-A, and the results with the more lipophilic ring-fluorinated analogs appear to support this view [K. L. Kirk, with D. Murphy, J. Costa, and N. Bartek (NIMH)].

In studies designed to investigate the structural features necessary for the transport and storage of 5-HT, it has been found that 4,6-difluoro-5-HT is taken up by human platelets at a much higher rate than 5-HT itself. Since the fluorine substituents increase the acidity of the phenolic group without perturbing the basicity of the primary amine, these results appear to support the hypothesis that the zwitterionic form of 5-HT is the actual substrate being transported. [K. L. Kirk, with J. Costa (NIMH)].

Ring-fluorinated analogs of dopamine (2-, 5-, or 6-fluoro) have been evaluated for dopamine activity in vivo (dogs); despite their increased phenolic acidity, all these analogs show activity essentially identical to that of dopamine, suggesting phenol acidity to be a noncritical factor in dopamine activity. The similarity in activities may be more apparent than real, however, since decreased activity could be counterbalanced by more rapid uptake (see above). Appropriate experiments with isolated receptors are in progress. [K. L. Kirk, with C. R. Creveling (NIAMDD), L. I. Goldberg (U. Chicago), and G. Firnau (McMaster U.)].

Detection of Fluorine in Tissues by Electron Energy-loss Spectroscopy

Electron energy-loss spectroscopy, an efficient method for the detection of low levels of elements below calcium in the periodic table, has been applied for the first time to detect and localize fluoro analogs in biological materials. We had shown previously that tritiated 4,6-difluoroserotonin is taken up efficiently by human platelets with concomitant displacement of endogenous serotonin. By use of the new spectroscopic technique on air-dried platelet whole mounts, the fluoro analog was localized in the platelet storage vesicles, providing direct evidence that serotonin had been stored in and released from the same vesicles. The combination of electron energy-loss spectroscopy and fluorine as a tracer offers several advantages for substrate localization in tissues: a high signal-to-noise ratio, the virtual absence of random fluorine in living cells, and the frequent simulation of natural substrates by their fluorinated analogs. [K. L. Kirk, with J. Costa (NIMH), D. C. Joy and D. M. Maher (Bell Labs.), and S. W. Hui (Roswell Park)].

Stereopopulation Control and Models for Enzyme Action

Studies have continued on the test-tube simulation of enzyme-substrate complexes and enzyme catalysis. A series of o-mercaptophenylpropionic acids have been synthesized and studied as models for sulfhydryl proteases. Kinetic studies show ring closure of the thiophenols to have a markedly different pH dependence than that for the analogous phenols, suggesting some mechanistic differences between serine and cysteine proteases. As in previous series, conformational freezing produces large rate-enhancement effects and shows that the free energy of formation of water is adequate to drive the formation of high energy bonds in biological systems. Unprecedented rearrangements have been encountered during the synthesis of these model compounds, while other novel rearrangements were found to occur during the synthesis of conformationally frozen imidazole systems. Kinetic studies on the hydrolytic ring opening of cyclic acylimidazoles revealed the rare phenomenon of buffer catalysis of both steps in the hydrolytic process, with buffer concentration determining the rate-limiting step. [L. A. Cohen, M. Blum, J. C. Reepmeyer].

Nmr studies with fluorinated compounds have shown conformational freezing to be even more extensive than orgianally assumed and computer-assisted calculations (CAMSEQ) indicate rotational barriers of 20 kcal or more in these super-reactive molecules [L. A. Cohen, M. M. King, M. Blum].

## SECTION ON CARBOHYDRATES

### Purification of Immunoglobulins

A general method for the purification of all IgA's has been worked out, including those of unknown specificity (M.K. Das, A.K. Bhattacharjee and C.P.J. Glaudemans).

### Binding Studies on Immunoglobulins and Preparation of Hybrid Recombinants

Anti-inulin IgA's have been studied for their binding activities. It has been found that they have a wide cylindrical cleft capable of interacting with the bulky α-helix-like 2→1 fructan. This cleft would bind the narrower ribbon-like 2→6 fructan as well, and thus a possible explanation is found for the fact that all anti-inulins also bind levans, but not vice-versa. The binding site of E109, A4, A47N and U61 can accommodate 3-4 fructose units. Binding seems to be dominated by the L-chain (D.G. Streefkerk and C.P.J. Glaudemans), indicated by the study of hybrid recombinant IgA's.

Anti-galactan hybrid recombinant IgA's show properties derived from the H-chain donor (B.N. Manjula and C.P.J. Glaudemans).

### Antibody Response to Phosphorylcholine (PC) Containing Antigens

The inbred Balb/c mouse responds to PC-antigens by a very limited expression of its clonal proliferation. This is in contrast to the non-inbred horse which shows a much higher specificity for the actual antigen used as an immunogen (B.N. Manjula and C.P.J. Glaudemans).

### Synthesis of Fluorescent Saccharides

4,5 Dimethylaminonaphthalenesulfonate derivatives of mono- and oligosaccharides containing glucose and galactose have been prepared. These derivatives can be used to study the binding of ligands to immunoglobulins with greater sensitivity (E. Zissis and C.P.J. Glaudemans).

## SECTION ON MEDICINAL CHEMISTRY

### Testing for Analgesic Activity, Toxicity, Dependence Liability and Narcotic Antagonism

This project is designed for "base-line" studies for analgesic activity by the hot-plate and Nilsen methods (subcutaneous and oral administration) and acute toxicity for more interesting compounds. These more promising com-

pounds are further assessed for physical dependence and antagonistic properties in Rhesus monkeys. Occasionally self-administration experiments (for psychologic abuse potential) are performed. Approximately 143 new compounds from universitites, the Laboratory of Chemistry and the (world-wide) pharmaceutical industry were submitted to this program and tested. About 50 compounds were assessed for physical dependence liability and antagonistic property. The reports on thebaine, a rather toxic compound obtained from Papaver bracteatum are of special interest. It will be studied more intensively (A. E. Jacobson and E. L. Atwell).

Theoretical, In Vivo and In Vitro Study of Analgesics and their Antagonists

Different types of analgesics and narcotic antagonists were examined by a variety of in vivo and in vitro techniques to discern their effect on the narcotic receptor. Brominated narcotic antagonists were prepared, as were agonist-antagonists with unusual substituents. In vivo and in vitro exploration of 3-bromoallylnormorphine will be continued. A theoretical study was completed to relate antinociceptive activty of diverse analgesics with their binding affinity to "opiate" receptors and their lipophilicity. An N-cyanoethylmorphinan was found to be about 100 times as potent as morphine and appeared to act like a narcotic antagonist in single dose suppression studies in monkeys. These compounds will be further examined by withdrawal studies and in primary addiction studies in primate species (A. E. Jacobson and M. F. Rahman).

Synthesis and Evaluation of Potential CNS, Antiinflammatory, and Anticancer Agents

A number of long acting narcotic antagonists in the 6,7-benzomorphan series were synthesized and evaluated in various in vivo and in vitro systems. Potential CNS agents were synthesized in the THC series and examined in several in vivo and in vitro systems. Conditions were found for N-demethylation of morphine to normorphine under which no dihydronormorphine is produced (K. C. Rice, A. E. Jacobson, M. Iorio and M. F. Rahman).

Synthesis and Pharmacology of (+)-Antipodes of Narcotic Agonists and Antagonists

In order to provide research tools to study the phenomena of narcotic analgesia, addiction and tolerance, synthesis of the (+)-antipodes of codeine, morphine, heroin and naloxone was undertaken. (+)-Codeine and (+)-morphine have now been prepared in multigram amounts and subjected to detailed pharmacological evaluation. Synthesis of (+)-heroin and (+)-naloxone is nearing completion and pharmacological evaluation will follow (K. C. Rice, I. Iijima, J. Minamikawa and A. Brossi).

The Synthesis and Pharmacological Examination of Retinoids

Retinal (Vitamin A aldehyde) and C-13 cis retinal have been utilized as starting materials to prepare known retinoids for studying the inhibition of carcinogenic processes (A. E. Jacobson and A. Brossi).

## Practical Total Synthesis of Racemic Codeine and Other Important Opium Alkaloids

A vastly improved synthetic method which does not require protecting groups and which is readily applicable to large scale work has been extended to a synthesis of a number of N-acyl norreticuline derivatives which are important precursors of opium alkaloids. Preliminary phenolic oxidation experiments have given racemic N-acyl salutaridine derivatives which can be readily converted to racemic codeine and other opium alkaloids using established methods (K. C. Rice, A. Brossi and M. F. Rahman).

## 3,4-Disubstituted Morphinanes

Structure-activity studies within a series of 3,4-substituted morphinanes of the (-)-series have shown that 3,4-dimethoxy-N-methyl morphinan is an interesting analgesic (A. Brossi and M. F. Rahman).

## Direct and Sequential Removal of Phenolic Oxygen Functions in Alkaloids

Phenolic groups in alkaloids can be removed by cleavage of their phosphate esters with alkali metals in liquid ammonia or by catalytic hydrogenation of their tetrazolyl ethers. Direct and sequential removal of present or newly created phenolic substituents in the series of aporphine alkaloids could be successfully completed. Apomorphine was thus converted into R-(-)-aporphine whereas S-(+)-bulbocapnine afforded its S-(+)-enantiomer (A. Brossi, K. C. Rice and M. F. Rahman).

## Condensation of Biogenic Amines with Carbonyl Compounds under "So-called" Physiological Conditions

Formaldehyde and acetaldehyde were the only carbonyl compounds which reacted with histamine, dopamine and serotonin in a buffered solution of pH 7.4 at 37°. No reaction could be observed with tryptamine and 6-hydroxydopamine (A. Brossi, M. Iorio and A. E. Jacobson).

## Molecular Features of Colchicine Associated with Antimitotic and Antigout Activity

Natural (-)-colchicine can easily be converted into trimethyl colchicinic acid (TMCA). The latter can be racemized in the form of its Schiff's base with KOH in methanol. Mescaline has been converted by standard procedures into a variety of N-trimethoxybenzyl and 1-trimethoxyphenyl substituted tetrahydroisoquinolines to be evaluated *in vitro* as antimitotic agents (A. Brossi and M. Iorio).

SECTION ON METABOLITES

## Synthetic Photochemistry

In collaboration with Masako Nakagawa of Chiba University, Japan, and with Isabella Karle, Nav. Res. Lab., the stereochemical configuration of a potential metabolite of tryptophan, isolated from the photooxydation of D,L- and L-tryptophan and characterized and crystallized as the N(1)-carbomethoxy-derivative, has been established as methyl 1-carbomethoxy-2,3, 3aα, 8aα-tetrahydropyrrolo[2,3-b]-indole-2β-carboxylate which on alkaline hydrolysis gave (cyclic) β-hydroxy-ψ-tryptophan, a presumable intermediate between tryptophan and kynurenine.

Another study with Prof. Y. Kanaoka, Hokkaido University, Japan, and Dr. Isabella Karle, NRL, showed that it is possible to disrupt certain aromatic systems by the photoaddition of methanol or ethanol. In the case of the irradiation of 2,4-dimethylphthalimide in methanol or ethanol the novel product was 2(N), 7β-dimethyl,7α-O-ethyl(Methyl)-1,3,3aβ,6,7,7aβ-hexa-hydroisoindol-1,3-dione, a versatile starting compound for novel hydroaromatic heterocycles. (Witkop)

## Interferon Induction by Synthetic Polynucleotides and the Antiviral Activity of Nucleoside Analogs

The structural and conformational features that govern interferon induction are investigated by the synthesis and evaluation of new polynucleotides. The biochemical basis of the observed stringent structural requirements for polynucleotide interferon inducers is sought by investigation of the cell membrane binding properties of nucleic acids and their ability to inhibit protein synthesis in a cell free system. The antiviral and antimetabolic properties of newly synthesized nucleoside analogs is also under investigation. (Torrence, Witkop, Huang, Edwards)

## Synthesis of Nucleoside Analogs with Possible Antiviral Properties

The synthesis of the alanosine derived nucleoside and nucleotide (Ia and Ib) is being pursued in order to determine its effect on adenosine biosynthesis and in order to elucidate the antiviral and antitumor action of alanosine itself. (Spande, Torrence)

## Central Nervous System agents based on the Piperidine ring system

Several compounds exhibited potency in the codeine-morphine range in the mouse hot-plate assay. Some activity has also been shown in the mouse p-phenylquinone abdominal stretch and tail-flick assays. In general, these esters showed no morphine-like physical dependence capacity in single dose suppression experiments in monkeys. Typical narcotic agonist or antagonist activities were absent. Binding to the opiate receptor *in vitro* was marginal or absent. (Waters)

## SECTION ON MICROANALYTICAL SERVICES AND INSTRUMENTATION

### Service Function and Instrumentation

Basic research and service functions are performed by members of the Section. A major mission of the organization involves the instrumental and chemical analyses provided to scientists of the Laboratory of Chemistry, NIH, and to a limited extent to personnel of other government agencies. Approximately 30 of the more common elements and five functional groups are determined on a quantitative basis, using ultramicro, micro and semi-micro techniques as required. The materials analyzed include organic and inorganic research samples, commercial preparations and various biological specimens. Molecular weights are determined by vapor pressure osmometry in both aqueous and non-aqueous solvents when requested. Instrumental analyses include: GC/MS spectrometry, mass spectrometry, nuclear magnetic resonance, atomic absorption spectrophotometry, ultraviolet, and flame photometry. Chemical analyses are done by most of the commonly used techniques including: gravimetric, colormetric, gasometric, coulometric, and volumetric. Assistance in the interpretation of spectra is rendered on request (David F. Johnson).

### Characterization of the Dehydrogenase-Isomerase Enzymes and Metabolic Pathways in Tetrahymena Pyriformis

Previous identification in this laboratory of 4-androstene-3β,17β-diol and pregnenolone as transformation products from the incubation of T. pyriformis with testosterone and progesterone, respectively, indicated that this protozoan possesses a dehydrogenase-isomerase system that can isomerize $\Delta^4$-steroid to a $\Delta^5$-steroid and reduce the C-3 carbonyl to a 3β-hydroxyl. The progesterone to pregnenolone transformation is the first demonstration of reversal of the 5-ene-3β-hydroxy ⇆ 5-ene-3-ketone ⇆ 4-ene-3-ketone pathway in an intact organism. Our objective is to characterize the nature of this dehydrogenase-isomerase system by purification of the dehydrogenase and isolation of pathway intermediates. A growth inhibition study will also be done to investigate the hypothesis that these transformations are related to the toxicity of steroids to this protozoan (Nancy S. Lamontagne).

### Application of NMR in Biochemical and Biological Systems

The purpose of this project is to develop nuclear magnetic resonance methods for elucidating molecular structure and for studying the interactions within and between molecules, especially those of biological interest. Various NMR methods (e.g., selective decoupling experiments, lanthanide shift experiments, etc.) have been successfully applied in a wide variety of investigations, including: assignment of structure to compounds such as the dihydrodiols derived from bacterial oxidation of the carcinogens benzo[a]pyrene and benzo-[a]anthracene, the halomorphides (narcotic drugs) and an unique galactoside from male drosophilie melanogaster; study of the interaction between phosphate containing molecules, 4-desoxypyridoxine phosphate and monomethyl phosphate, and a number of structurally related biogenic amines; study of the binding of cyanide ion, imidazole and the mixed ligand to ferriprotoporphyrin IX·Cl (hemin); study of [$^{15}$H]-$^{1}$H INDOR experiments to obtain the $^{15}$N-labeled meso-

tetraphenylporphrin; and study of iron-protein and iron-ligand bindings in hemerythrin, a nonheme iron protein responsible for oxygen transport in certain species of marine invertebrates (Herman J. C. Yeh).

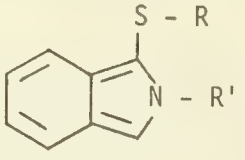
The Development of Chemical Methods and Compounds for the Study of Biological and Medical Problems

The primary goal of this work is to contribute to the investigation and solution of basic biological and medical problems. This is to be done by the development of chemical methods and reagents for the study of the primary, secondary, tertiary, and quaternary structures of biomacromolecules, by the study, development, and application of organic and photochemical reactions, and by synthesis of selected chemical compounds (Calvin M. Foltz).

Nature of Steroid-Receptor Interactions

The objective of this project is to define the initial, intra-cellular events of steroid hormone action. Synthetic glucocorticoid derivatives, some of which could potentially react to form covalent receptor-steroid complexes, are being used with glucocorticoid-responsive rat hepatoma tissue culture cells to examine: (1) steroid-receptor binding site interactions; (2) the effects of steroid binding on receptor conformation; (3) the nature of "activation" of receptor-steroid complexes and (4) the nuclear binding of activated receptor-steroid complexes. Fluorescence spectroscopy represents a novel technique for examining these interactions. For this reason, the fluorogenic reaction of o-phthalaldehyde and β-mercaptoethanol with primary amines has been investigated and the fluorescent products defined as previously unknown, 1-alkylthio-2-alkylisoindoles. Further studies have been directed at adapting these isoindoles for a fluorescent study of steroid-receptor interactions.

The fluorogenic reaction of o-phthalaldehyde (OPTA) and β-mercaptoethanol (MERC) with primary amines and amino acids has been examined because the hitherto undefined, intensely fluorescent products are detectable at concentrations $\leq 10^{-9}$M. The structure of these fluorescent products was found to be the previously unknown 1-alkylthio-2-alkylisoindoles I. While these compounds are unstable, the first stable member of this class of compounds




I

R = alkyl, aryl

R' = alkyl

(i.e., I where R = t-Bu, R' = n-Pr) has been isolated. The mechanism of the reaction, the nature of the intermediates, the scope and limitations of the reaction and the properties of the fluorescent products have all been examined. These studies have allowed for modification of the OPTA-MERC-amine reaction, and preparation of a more versatile fluorescent isoindole. Investigation of the feasibility of attaching these isoindoles to a glucocorticoid steroid,

which could permit a fluorescence study of steroid-receptor interactions is presently proceeding.

Alternative methods of studying receptor-steroid interactions are 1) to examine the effects of subtle modifications of a steroid hormone and 2) to prepare steroids capable of forming a covalent receptor-steroid complex. Several 21-substituted derivatives of cortisol have been prepared; but, this project is only in the beginning stages. Numerous other derivatives, both of cortisol and dexamethasone, are scheduled to be synthesized during the next year (S. S. Simons, Jr.).

SECTION ON OXIDATION MECHANISMS

Enzymatic Oxidation of Drugs to Toxic and Carcinogenic Metabolites

Previous annual reports from this Section have described a systematic approach to the study of the cytotoxicity, mutagenicity, and carcinogenicity of the ubiquitous environmental carcinogen benzo[a]pyrene (BP). Briefly these studies have consisted of i) synthesis of as many known and potential oxidative metabolites as was possible, ii) study of the metabolism of the hydrocarbon with these authentic standards in hand, iii) testing of these compounds for cytotoxic and mutagenic activity with bacterial and mammalian cells both in the presence and in the absence of added drug metabolizing systems such as cytochrome P-450 and epoxide hydrase, and iv) evaluation of the carcinogenicity of these synthetic standards in several animal models. The majority of these studies on benzo[a]pyrene are nearing completion.

Studies of the inherent mutagenic and cytotoxic activity of all twelve isomeric phenols, four of the six metabolically possible arene oxides and corresponding trans-dihydrodiols, and five quinones of BP have been examined in histidine dependent mutants of Salmonella typhimurium and 8-azaquanine sensitive Chinese hamster V-79 cells. The most mutagenic of these compounds was BP 4,5-oxide, although several of the phenols had weak but significant mutagenic activity. The phenols, dihydrodiols, and BP were subjected to metabolic activation with a cytochrome P-448 containing system in the presence of the bacteria with the result that BP 7,8-dihydrodiol was more strongly activated to mutagens than were the other compounds. This mutagenic activity was presumed due to the diastereomeric 9,10-oxides of the 7,8-diol. The mutagenic activity of these 7,8-diol-9,10-epoxides was not blocked by the presence of epoxide hydrase and the presence of the hydroxyl groups at the 7- and 8-positions was found to be nonessential to mutagenic activity. The 7,8-diol-9,10-epoxides are among the most mutagenic compounds yet tested in V-79 cells. With the conditions for metabolic activation established in the experiments with the BP derivatives, the five metabolically possible trans-dihydrodiols of benzo[a]anthracene (BA) were then examined. As anticipated from quantum mechanical calculations, the BA 3,4-dihydrodiol caused the highest number of mutations. This result provides the basis for a new theory of hydrocarbon-carcinogenicity which will be described later.

In the previous annual report, BP 7,8-oxide was described as strongly carcinogenic on mouse skin but weaker than BP. Study of the 4-, 5-, 6-, 7-, 8-, 9-, and 10-hydroxy BP on mouse skin has shown these compounds to be non-

tumorgenic under conditions where BP caused tumors in all the animals. These studies show that the carcinogenicity of BP 7,8-oxide is not due to its phenolic isomerization products and casts serious doubt on the theory that 6-hydroxy BP is an ultimate carcinogen from BP. BP 7,8-dihydrodiol which results from the action of epoxide hydrase on BP 7,8-oxide is a better skin carcinogen than BP 7,8-oxide and is equipotent to BP. However, in the newborn mouse, BP 7,8-dihydrodiol is much more tumorgenic than BP and can be considered as a proximate carcinogen. On mouse skin, the 7,8-diol-9,10-epoxides and 9-hydroxy BP are potent agents in causing hyperplasia.

Convenient synthetic methods have been devised for the preparation of specifically tritiated arene oxides such that these compounds could be examined as substrates for the glutathione transferases and epoxide hydrase. The most rewarding of the enzymatic studies have been those with epoxide hydrase in which a highly sensitive and rapid thin layer chromatographic assay was developed which showed that phenanthrene 9,10-oxide was one of the best known substrates for the enzyme. In addition, BP 4,5-oxide was found to be the most sensitive substrate for assaying small amounts of activity. Studies with optically active cis-stilbene oxides established that the enzyme has a marked preference for attack at an S-center in epoxide substrates. Studies with human livers established that the substrate requirements of both the human and rodent derived enzyme are very similar.

Studies of the metabolism of BP by microsomes from the lungs of inbred strains of mice which vary markedly in their susceptibility to chemical carcinogens showed very little in the way of differences in the profile of metabolites. There were, however, markedly different levels of control and carcinogen-induced activity for these strains. Examination of the metabolism of BP 7,8-dihydrodiol by liver microsomes and reconstituted drug metabolizing systems has shown dramatic stereospecificity and regiospecificity. In all cases, the dihydrodiol is converted into a mixture of the two diastereomeric 7,8-diol-9,10-epoxides. The (-)-enantiomer of the dihydrodiol is converted predominantly into one diastereomer while the (+)-enantiomer is converted preponderantly into the other diastereomer. Enzyme preparations from the livers of non-induced animals also form large amounts of 6-hydroxylated dihydrodiol. Studies of the combined stereospecificity of the cytochrome P-450 system and epoxide hydrase have indicated that (-)-BP 7,8-dihydrodiol is the predominant enantiomer formed from BP. This specificity is due to stereospecificity by the cytochrome P-450 system rather than epoxide hydrase. This result is of key significance to further carcinogenesis testing.

Since a mounting body of evidence has indicated that the 7,8-diol-9,10-epoxides of BP are ultimate carcinogens and since their in vivo formation occurs with enantiomeric specificity, the two pairs of enantiomeric diastereomers have been synthesized, and their absolute stereochemistry has been assigned by optical methods. Comparison of the products formed between these four compounds and poly(G) in vitro with the RNA-hydrocarbon adducts formed when BP is applied to mouse skin have shown that one enantiomer of each diastereomer is bound to RNA in vivo. These diol epoxide adducts are derived from both enantiomers of BP 7,8-dihydrodiol. Chemically, these RNA adducts are formed by attack of the N-2 amino group of guanine at C-10 of the diol epoxides. Whether or not this structural modification of RNA or a similar one in DNA

plays a role in carcinogenesis remains to be examined. Both of the diol epoxide diastereomers bind to the phosphate backbone in nucleic acid and thereby induce strand breaks.

In an attempt to generalize the concept of diol epoxides as ultimate carcinogens from polycyclic aromatic hydrocarbons, we have conducted quantum mechanical calculations which should predict reactivity for these compounds. These calculations predict which of the several diol epoxides of a given hydrocarbon should be most reactive and allow the hydrocarbons to be ranked for carcinogenicity based on the most reactive diol epoxide. In essence, these calculations require that the epoxide form part of a bay-region of the hydrocarbon (typified by the 4,5-region of phenanthrene) and provide a reasonable ranking of the hydrocarbons for carcinogenic activity. In order to test the bay-region theory, the five dihydrodiols of BA were synthesized and tested for mutagenic activity on metabolic activation. As predicted, the 3,4-dihydrodiol, which can be converted into a 1,2-epoxide (bay-region), was the most active. Diol epoxides of the BA dihydrodiols have been synthesized to expedite further mutagenesis testing.

The above studies have been conducted primarily by Drs. D. M. Jerina, H. Yagi, D. R. Thakker, H. D. Mah, R. E. Lehr, and M. Schaefer-Ridder of this laboratory. A portion of the studies were done in collaboration with Drs. A. H. Conney, W. Levin, and A. W. Wood at Hoffmann-La Roche. A total of 46 publications appeared, some of which were the result of efforts by previous workers in this Section.

## SECTION ON PHARMACODYNAMICS

### Pharmacologically Active Compounds from Tropical Frogs and Other Natural Sources

Further quantities of dendrobatid alkaloids have been isolated for determination of structure and investigation of their unique pharmacological properties. Gephyrotoxin, an active muscarinic antagonist was shown to be a unique tricyclic alkaloid by X-ray crystallography. In toto, nearly 100 different alkaloids have been detected and characterized in extracts from over thirty different species of dendrobatid frogs. These alkaloids have been divided into six different classes and structures assigned to the majority. A 2,6-disubstituted piperidine has been detected in one species and perhaps represents a precursor class for the biosynthesis of the bicyclic histrionicotoxins and pumiliotoxins. Certain disubstituted piperidines were synthesized for pharmacological investigation. The dendrobatid alkaloids are proving invaluable to the definition of intraspecific, intrageneric, evolutionary, and ecological relationships in this family of frogs. As yet they have not been detected in any other family of frogs. A small neotropical toad of the genus Dendrophryniscus was found to contain unique lipid soluble neutral compound with long-lasting sedative activity (Daly).

A water soluble toxin was found in significant amounts in roe and liver from a fish of the Red Sea. The properties of this toxin are very much like those of tetrodotoxin. Extracts of engorged ticks from Colorado contained only very low levels of a paralytic toxin (Daly).

Batrachotoxin did not cause depolarization of cells in the innervated membrane of the electroplax of the electric eel. If the membrane was partially depolarized by electrical stimulation, carbamylcholine, or high concentrations of potassium, batrachotoxin caused a complete and relatively irreversible depolarization. The results indicate that the interaction of batrachotoxin with action potential sodium channels is strongly voltage dependent (Daly).

Radioactive perhydrohistrionicotoxin bound reversibly and with high affinity to a limited number of sites in membranes from the electroplax of the Torpedo fish. After solubilization the perhydrohistrionicotoxin-binding site could be separated from the acetylcholine receptor. The data were, in toto, consonant with the presence of perhydrohistrionicotoxin-binding sites on the ion conductance modulator of the acetylcholine receptor. The modulator and receptor appear to be readily dissociable entities. Interaction of histrionicotoxins with this site on the modulator is responsible for blockade of responses to acetylcholine (Daly, Witkop).

Dihydroisohistrionicotoxin noncompetitively inhibited binding of scopolamine to muscarinic receptors in neuroblastoma cells and neuroblastoma-glioma hybrid cells. Tetracaine had similar effects on the binding of scopolamine (Burgermeister).

Cultured chick-embryo skeletal muscle cells possess a high concentration of nicotinic acetylcholine (ACh) receptors. Stimulation of these receptors with 1 mM carbamylcholine greatly increases the ion permeability of the cells. The cultured cells have provided a valuable system for studying the effects of dihydroisohistrionicotoxin (DHTX) on (i) the sodium transport activity of the ACh receptor, and (ii) the affinity of the ACh receptor for cholinergic ligands. DHTX inhibits the ACh receptor-mediated uptake of sodium into the cells with an $IC_{50}$ of 0.5 μM. Binding of DHTX to the ACh receptor occurs only at a much higher concentration of the toxin as was demonstrated by its ability to inhibit binding of [$^{125}$I]α-bungarotoxin to the receptor ($IC_{50}$ = 30 μM). Further studies have shown that DHTX has an allosteric effect on the ACh recognition site. Binding of the ACh receptor agonists, carbamylcholine, ACh, decamethonium, nicotine, and the ACh receptor antagonists, d-tubocurarine, and gallamine, was measured by inhibition of [$^{125}$I]α-bungarotoxin binding to the ACh receptor of the cells. In the presence of a very low concentration of DHTX binding of the agonists was increased whereas binding of the antagonists was unchanged. The $IC_{50}$ values of the agonists were reduced to 1/10 to 1/20 of the $IC_{50}$ values observed in absence of DHTX. By measuring the initial rate of α-bungarotoxin binding in absence and presence of carbamylcholine and DHTX, it was found that the change of receptor affinity for carbamylcholine occurs within the first 5 minutes of incubation. Affinity changes of equal size and rate can be produced by either 5 μM carbamylcholine plus 1 μM DHTX or 40 μM carbamylcholine without DHTX. When the cells are preincubated with 40 μM carbamylcholine and then stimulated with 1 mM carbamylcholine, the ACh receptor-mediated sodium transport is progressively reduced. This phenomenon, which is caused by desensitization of the ACh receptor, shows a very similar time dependence as the receptor affinity change for carbamylcholine produced by either 40 μM carbamylcholine or 5 μM carbamylcholine plus 1 μM DHTX. The following hypothesis is proposed for the action of DHTX. The toxin binds to a site allosteric to

the ACh recognition site of the ACh receptor. Thereby a conformational transition of the ACh receptor is caused. The resulting ACh receptor conformation represents a desensitized state since it does not mediate sodium transport. It differs from the resting state of the ACh receptor by having a higher affinity for receptor agonists (Burgermeister).

Derivatives of pseudo-pelletierine (N-methyl-granatanone) which have structural similarities with histrionicotoxin have been synthesized by N- and C-alkylations. N-pentyl and N-benzyl derivatives of pseudo-pelletierine and the two corresponding alcohols of each (3α- and 3β-) have been synthesized. The addition of n-pentyl to the carbonyl group was impossible with the Grignard reagent because of the low reactivity of pseudo-pelletierine, but the analogous reaction with lithium and n-bromopentane was successful. Alkylation of N-pentyl and N-benzyl derivatives of pseudo-pelletierine should yield the di-alkylated pseudo-pelletierines. Preliminary results indicate that $5 \times 10^{-5}$ M N-methyl,3-pentyl-granatan-3β-ol, like histrionicotoxin and like dihydroadaline (a related alkylated pseudo-pelletierine), inhibits sodium uptake elicited by carbachol by 50% in chick muscle cells (Van Dorsselaer, Burgermeister).

## The Role of Cyclic Nucleotides in the Nervous System

The antagonist activity of clonidine and various analogs at norepinephrine-sensitive cyclic AMP-systems in rat brain slices showed no correlation with their activity as α-agonists in peripheral systems. Clonidine antagonized amphetamine-excitation in rats and it was proposed the antagonism of α-adrenergic agonist-sensitive cyclic AMP-generating systems by clonidine and phentolamine provides a possible basis for the similar central depressant activity of these two compounds which are normally considered an α-agonist and an α-antagonist respectively (Skolnick).

The effect of various agonists and antagonists on α-adrenergic agonist-sensitive adenylate cyclase in rat brain slices was shown to correlate with their effects on binding at the α-antagonist, WB 4101, to rat brain membranes. It was proposed that this compound is a useful tool for the study of α-sensitive cyclic AMP-systems in brain (Skolnick).

Tazolol, a reported β-agonist in heart, was shown to be a potent β-antagonist of norepinephrine-elicited accumulations of cyclic AMP in rat brain slices and a potent inhibitor of the depressant effects of norepinephrine on firing of central neurons (Skolnick).

The denervation-induced hyperresponsiveness of norepinephrine-sensitive cyclic AMP-generating systems in brain has been investigated in detail with two strains of rats. 6-Hydroxydopamine was used to disrupt noradrenergic input. In Sprague-Dawley rats both α- and β-responses were enhanced by about 2 fold after 6-hydroxydopamine, while in Fisher 344 rats, the noradrenergic responses which were already relatively high in control animals were not further increased by 6-hydroxydopamine-treatment. Binding at a β-adrenergic ligand, dihydroalprenolol was enhanced by about 25% after 6-hydroxydopamine in both rat strains. Binding of an α-adrenergic ligand, WB 4101, was unchanged. Thus, levels of receptors do not correlate with changes in responsiveness of cyclic AMP-systems. Instead, data with homogenates suggest that alterations in adenylate cyclase

activity may account for the adaptive changes in Sprague-Dawley rats after 6-hydroxydopamine (Skolnick).

Estrogen-elicited accumulations of cyclic AMP in incubated intact hypothalmi had, in earlier studies, been shown, based on antagonist paradigms, to be mediated by catecholamines. Further support for catecholamine as intermediates was provided by data demonstrating that turnover of catecholamines is enhanced after prolonged incubation of hypothalami with diethylstilbestrol. Catechol estrogens were found to inhibit estrogen-elicited accumulations of cyclic AMP in hypothalami of female rats, apparently by competition at an estrogen binding site. The results strongly suggest that 2-hydroxyestradiol is an endogenous antiestrogen (Skolnick).

Histamine-elicited accumulations of cyclic AMP in guinea pig cerebral cortical slices involved both $H_1$- and $H_2$-histaminergic receptors. The $H_1$-response was greatly potentiated by adenosine and was dependent on the presence of extracellular calcium ions. These results parallel prior results with norepinephrine-sensitive cyclic AMP-generating systems in brain slices where the α-response was found to be potentiated by adenosine and to be dependent on extracellular calcium. A vesicular or "microsac" preparation from rodent brain showed both $H_1$ and $H_2$-responses similar to those of brain slices and is being further investigated (Creveling).

During isolation of calcium-dependent activator protein from rat brain, a number of heat-stabile inhibitors and activators of phosphodiesterases were isolated. At least three factors were present which inhibited the calcium-dependent phosphodiesterase. Two were low molecular weight protease-insensitive compounds. These low molecular weight compounds appeared to be potent inhibitors for phosphodiesterases in a "microsac" preparation from brain. At least two protein factors were present which inhibited calcium-independent phosphodiesterase. At least four protein factors were present which activated high Km cyclic AMP-phosphodiesterase (Kanamori).

Calcium-dependent activator protein was found in brain slices to be present both membrane-bound, presumably associated with adenylate cyclase, and in the cytosol, presumably associated with phosphodiesterase. The effects of stimulation of cyclic AMP-generating systems on the subcellular distribution of activator protein in brain slices is under investigation (Davis).

Deoxycoformycin and erythro-9-(2-hydroxy-3-nonyl)adenine have been shown to be potent inhibitors of rat brain adenosine deaminase. The deoxycoformycin potentiates amine-elicited accumulations of cyclic AMP in rat brain slices, apparently by protecting endogenous adenosine from enzymatic deamination (Nimitkitpaisan).

Pharmacodynamic Amines and Enzymes Involved in Their Metabolism

Studies on the mechanism by which 6-hydroxydopamine and 5,6-dihydroxytryptamine and related compounds induce a specific neurotoxicity *in vivo* have been continued. An oxygen-dependent binding and subsequent cross-linking of model proteins was demonstrated. Stoichiometric measurements of this binding and other properties indicate that the binding and cross-linking occurred pri-

marily through free sulfhydryl groups on protein. Attempts have been made to relate this model for cytotoxicity to events in neuroblastoma cultures, isolated mouse atrial preparations, mitochondrial preparations, and in intact animals. A rationale for the design of new cell-specific toxic agents was developed based on these studies. While the present results indicate a relationship between the irreversible binding of neurotoxic amines and the cytotoxic sequela, no direct evidence for amine-elicited polymerization in vivo has been obtained (Creveling).

The mechanism of cytotoxicity of 5,7-dihydroxytryptamine differs substantially from that of both 5,6-dihydroxytryptamine and 6-hydroxydopamine and its cogeners in that it fails to bind to model proteins in vitro and its action in vivo is blocked by prior treatment with monoamine oxidase inhibitors. Studies with isotopically labelled 5,7-dihydroxytryptamine both in vitro and in vivo indicated that the mechanism of its cytotoxic action could be subsequent to the enzymatic formation of an aldehyde intermediate (Creveling).

A non-neuronal, cytotoxic effect of 5,6-dihydroxytryptamine leads to the development of a time and dose-dependent development of subepicardial lesions of the myocardium. These lesions were preceded by a fatty infiltration of myocardial fibers. There was no apparent relationship between the specific neurotoxicity and the effects on the myocardium. Studies on the uptake and cellular and subcellular distribution of 5,6-dihydroxytryptamine indicated that there is a relatively large component of the uptake into non-neuronal tissue in heart preparations. The observed uptake was temperature-dependent and essentially irreversible. The nature and site of this binding phenomenon is under continuing investigation (Creveling).

Studies on the inhibition of norepinephrine uptake and the release of norepinephrine both in vivo and in isolated atrial slices are being continued to find additional compounds which may be useful as investigational tools. The kinetic parameters of the uptake mechanism are currently under study as a model for investigating the mechanism responsible for the transport of amine through the neuronal membrane. The transport and storage of the fluorotyramines, dopamines, and indoleamines in atrial preparations are being examined. The isolated atrial system in vitro is being used as a model to measure the release of amines stored intraneuronally. The possible involvement of S-adenosyl-L-methionine-dependent carboxymethyltransferase in the release of amines is being examined through the use of product inhibition of the transferase. (Creveling)

The cellular localization of catechol-O-methyltransferase was determined through the application of immunoperoxidase techniques using an antisera specific for the soluble form of the enzyme from liver. The study revealed an important localization of the enzyme not only in liver and kidney tubules but also in the reproductive organs of both male and female rat. Further, the presence of the enzyme was described in the spleen, bone marrow, lymphatics, and thymus. The results suggest that catechol-O-methyltransferase is important in the inactivation of steroidal catechols (Creveling).

A study of the kinetics of the inhibition of catechol-O-methyltransferase by a specific antibody preparation for this enzyme was carried out. This study

indicated that the antigenic sites differed from the catalytic sites on the enzyme molecule (Creveling).

A study of the cellular localization of catechol-O-methyltransferase in rat brain using an antibody for the soluble form of the enzyme isolated from rat liver by an immunofluorescent technique revealed the pressure of "glial"-like, enzyme-positive cells and a fine network of what appear to be dendritic processes (Creveling).

The intracellular localization of catechol-O-methyltransferase is being investigated with immunological techniques. The results indicate that the enzyme is present on rough coated particles, reticular endothelial structures, and perhaps plasma membranes. However, technical difficulties make a definitive localization at the ultrastructural level impossible at present (Creveling).

A study of the toxicity and histopathology of 2-fluorohistidine have demonstrated an interesting, apparently specific effect of this analog of histidine upon blood forming organs. A non-lethal dose of 2-fluorohistidine results in a time and dose dependent leucopenia, regression of the spleen, thymus, lymph nodes, and a profound hypocellularity of bone marrow. This effect has been explored as a therapeutic tool in leukemic mice with some success. The specific nature of the 2-fluorohistidine-effect is being investigated in various leukemia models in vivo, in cultured murine leukemia cells, and in normal mice (Creveling).

Studies on the metabolism of 2-fluorohistidine and a comparison to the metabolism of histidine are in progress. The present evidence indicates that 2-fluorohistidine substitutes in a competitive fashion with histidine for histidine amino acyl transferase resulting in the incorporation of 2-fluorohistidine into protein. 2-Fluorohistidine is a substrate for histidine ammonia lyase and gives raise to 2-fluorourocanic acid. The further metabolism is at present unknown but the present evidence suggests a direct entrance into tetrahydofolic acid metabolism (Creveling).

A collaborative project with NIDA to develop a new technique for the evaluation of animal behavior has been explored. The system involves the point to point tracking of one to six animals in an open field. The location of each animal, via a color television camera, is determined 10 times a second and information recorded and formated by an on-line computor onto a high speed tape. Software systems have been developed to analyze the motion patterns. This system is without precedent, necessitating a trial and error procedure for evaluating the significance of the selected patterns. At present, a set of thirty parameters which are designed to compare the interaction of two mice appears to be a promising avenue of approach. Studies were carried out with a variety of conditions to test the system. These included eight strains of mice, male-female comparisons, environmentally "deprived" vs environmentally enriched rodents, mice treated at birth with 6-hydroxydopamine and compared with untreated controls over a period of 8 months, animals treated with a variety of psychoactive drugs, and animals with specific amine acid deficiencies. These studies were done simultaneously with studies on amine-sensitive cyclic AMP-generating systems in rodent cortex (Creveling, Daly).

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PERIOD COVERED
July 1, 1976 to September 30, 1977

TITLE OF PROJECT (80 characters or less)

Studies on the Synthesis of Carbohydrate Derivatives for Biomedical Research

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

| | | |
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| PI: | | |
| R.K. Ness | Research Chemist | LC NIAMDD |

COOPERATING UNITS (if any)

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| C.P.J. Glaudemans | Chief, Sec. on Carbohydrates | LC NIAMDD |
| D. G. Streefkerk | Visiting Fellow | LC NIAMDD |

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Section on Carbohydrates

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20014

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SUMMARY OF WORK (200 words or less - underline keywords)

This project has been discontinued.



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July 1, 1976 through September 30, 1977

TITLE OF PROJECT (80 characters or less)

Immunochemistry and Reactions of Carbohydrates

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

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SUMMARY OF WORK (200 words or less - underline keywords)

How specific are anti-carbohydrate immunoglobulins? What is the nature of the combining site? To answer these questions the interactions of homogeneous immunoglobulins with carbohydrate haptenic groups will be studied. Site mapping will be studied through impact affinity labeling of the same globulins. Hybrid immunoglobulins using H,L recombinants are studied to define subunit interactions in myeloma protein. The requirements for immunogenicity in antigens are studied.

## Project Description

Objectives: Homogeneous immunoglobulins can be obtained from plasma tumors. The study of their interaction with polysaccharide antigens will reveal much about the nature of action of antibodies and about their structure. We are involved in such studies.

Methods Employed: The usual techniques of chemistry and biochemistry are employed, such as affinity chromatography employing hapten-coupled sepharose columns, fluorescence titration, organic synthesis, gas-liquid chromatography, mass spectroscopy, etc.

## Major Findings

1. General method for the purification of homogeneous immunoglobulins A.

Immunoglobulins are optimally purified by affinity chromatography, that is immunoadsorption on an inert support, followed by elution with soluble hapten. Since it is not always feasible to obtain the proper ligand it has become necessary to search for methods alternative to affinity chromatography, which may be used for Ig-purification. A general method has been developed (M.K. Das and C.P.J. Glaudemans). The myeloma proteins, after precipitation with ammonium sulfate were absorbed on a column of DEAE-Sephadex A25 and unwanted material removed by PBS washing. Phosphate buffer pH 8.0, and higher salt concentration, then yielded a partially purified IgA. Mild reduction and alkylation then gave monomers, which were separated by gel filtration on two subsequent columns of G-200 Sephadex. The IgA thus obtained showed a single band on immunoelectrophoresis against goat anti-whole mouse serum. Murine myeloma proteins MOPC 384 and 870 (unknown specificity) were thus purified. Both bind phosphorylcholine, but it appears that the site is located deep into the combining area (A.K. Bhattacharjee and C.P.J. Glaudemans). Further work is in progress to elucidate the specificity of these immunoglobulins.

2. Binding studies and evaluation of hybrid recombinant immunoglobulins.

a. Anti-inulins.

For immunoglobulins each variable domain can be regarded as made up of a fairly invariant amino acid framework upon which the hypervariable region are looped, thus creating a particular ligand binding specificity (work by, among others, E.A. Kabat, D.R. Davies and E.A. Padlan). The hypervariable segments of both the Heavy (H) and Light (L) chains thus constitute the major input leading to diversity of specificity among immunoglobulins. In our work on recombinant hybrid immunoglobulins with anti-galactan specificity, we have shown that the fluorescence behavior of the hybrids closely paralleled

that of the H-chain donor (see also below). This perhaps suggests that variance in the H-chain amino acid sequence mostly dictates the varying affinities for β(1→6)D-galactosyl ligands within this group of immunoglobulins.

It has been shown by x-ray diffraction studies (D.R. Davies' group at the NIH) on McPC 603 (an immunoglobulin with anti phosphorylcholine (PC) specificity) that, although the combining cavity is lined by the hypervariable region of both H and L chains, interactions between phosphorylcholine (PC) and the protein are mostly mediated by the H-chain. There is no reason to assume that H-chain dominance exists in general in determining the degree of affinity of a certain immunoglobulin with its ligand within a class of immunoglobulins having the same general binding specificity. Indeed, examination of the amino acid sequences by M. Potter's group (NCI) of a number of homogeneous immunoglobulins with anti (2→1)β D-fructofuranan specificity have revealed that there is, strikingly, near identity among the H-chains of ABPC4, UPC61, ABPC47N and EPC109, while the hypervariable regions of these proteins are identical. Thus, if these immunoglobulins are to bind ligands with individual affinity, this individuality has to arise from differences in the amino acid sequences of the L-chain of these immunoglobulins. To date, partial analyses of the L-chains by the same group has already revealed large differences in sequence, and it has thus become likely that, among homogeneous immunoglobulins with anti-inulin specificity, the L-chains exert a dominant influence on the degree of affinity between protein and polysaccharide antigen. It fortuitously turns out that in the class of anti-inulins, amino acid sequencing appears much more facile than in the homogeneous myeloma protein having anti-galactan specificity. In other laboratories progress is being made in x-ray diffraction studies of anti-inulin, conformational studies based on amino acid sequencing of these proteins, and on studies illuminating the relationships between secondary structure and idiotypes (R. Lieberman, NIH). In view of this the research in our own laboratory has recently been focussed on mapping of areas of interaction of oligosaccharide ligands with the antigen combining site of these murine immunoglobulins (see below); similarly in approach to the method used by us for the study of the galactan-antigalactan systems (see previous reports).

The immunoglobulins with anti-inulin specificity represent such a large and interesting class. Therefore, using the method of ligand altered fluorescence of immunoglobulins, we have studied the binding of oligosaccharides containing (2→1)-linked β-D-Fruf residues terminated by a (2→1)-linked α-D-Glcp moiety (thus corresponding to fragments of inulin) with mouse myeloma immunoglobulins A4, A47N, U61 and E109. Inulin itself was included in the study. ($F_1G_1^1$ is sucrose, $F_2G_1^1$, the 2→1 linked trisaccharide 1-kestose, etc.).

The heavy chains of these immunoglobulins have been analyzed for their amino acid sequence (M. Vrana, personal communication). All four immunoglobulins show the presence of tryptophan in, and near, the first hypervariable (hv) region of the heavy chain (H33 and H37), as well as tyrosine in the first and second hv region of the heavy chain (H32 and H58). These

TABLE 1

Maximum, ligand-induced changes in fluorescence (in %) of the myeloma proteins E109, A47N, A4 and U61, monitored at 340 nm after excitation at either 295 or 280 nm.

| Ligands | E109 | | | | A47N | | | |
|---|---|---|---|---|---|---|---|---|
| | monomer | | Fab' | | monomer | | Fab' | |
| | 295 | 280 | 295 | 280 | 295 | 280 | 295 | 280 |
| Me α-D-Glcp | -2.7 | --- | -0.8 | -0.7 | -2.1 | -0.6 | -1.2 | -1.2 |
| Me β-D-Fruf | -1.8 | -2.0 | -0.6 | -2.1 | -4.7 | -4.3 | -3.2 | -5.2 |
| Sucrose ($F_1G_1^1$) | -7.9 | --- | --- | --- | -4.2 | --- | --- | --- |
| 1-Kestose ($F_2G_1^1$) | -3.4 | --- | --- | -6.5 | -4.5 | --- | --- | -6.1 |
| Nystose ($F_3G_1^1$) | -2.1 | -6.5 | --- | -7.3 | -2.7 | -6.5 | --- | -6.6 |
| $F_4G_1^1$ | ∿0 | -6.6 | --- | -6.7 | <-1 | -4.8 | --- | -4.3 |
| Inulin ($F_nG_1^1$) | -3.7 | --- | -4.1 | --- | --- | --- | -5.7 | --- |

(Table 1 continued)

| Ligands | A4 | | | | U61 | | | |
|---|---|---|---|---|---|---|---|---|
| | monomer | | Fab' | | monomer | | Fab' | |
| | 295 | 280 | 295 | 280 | 295 | 280 | 295 | 280 |
| Me α-D-Glcp | -2.4 | -0.3 | -1.1 | -1.0 | -1.2 | ∿0 | -0.9 | -0.6 |
| Me β-D-Fruf | -1.8 | -2.2 | -2.6 | -6.3 | -0.5 | <-1 | -0.4 | -2.0 |
| Sucrose ($F_1G_1^1$) | -7.5 | --- | --- | --- | -4.5 | -7.2 | --- | -7.4 |
| 1-Kestose ($F_2G_1^1$) | -6.2 | --- | --- | --- | -1.4 | -2.7 | --- | --- |
| Nystose ($F_3G_1^1$) | -2.6 | -6.0 | -1.9 | -7.7 | <-1 | -2.5 | --- | -2.8 |
| $F_4G_1^1$ | --- | -7.5 | --- | -6.3 | --- | -3.9 | --- | -2.8 |
| Inulin ($FnG_1^1$) | --- | --- | -4.9 | --- | <-2 | -9.1 | --- | -5.2 |

residues may be involved in the ligand-induced altered fluorescence we have observed for these four proteins.

Table 1 lists the maximum changes (decreases) in fluorescence ($\Delta F_{max}$) for the monomeric myeloma IgA proteins A4, A47N, E109 and U61 as well as for their Fab' fragments, induced by the following ligands: methyl α-D-Glcp, methyl β-D-Fruf, sucrose ($F_1G_1^1$), 1-kestose ($F_2G_1^1$), nystose ($F_3G_1^1$) and the pentasaccharide $F_4G_1^1$. The $\Delta F_{max}$ for the four Fab' fragments in the presence of excess inulin is also given.

Ouchterlony double diffusion experiments showed that of the four monomeric immunoglobulins, only E109 failed to precipitate with inulin. Measurement of the $\Delta F_{max}$ values (and also the association constant) for any monomeric protein with inulin would obviously be fruitless if this is a precipitating system. A measurement of ΔF as a function of inulin added to an immunoglobulin is only meaningful in the case of E109. Indeed, the $\Delta F_{max}$ for this system (see Table 1) is only slightly lower than for the system E109 (Fab')-inulin.

Table 2 lists the association constants Ka for the four monomeric immunoglobulins and for their Fab' fragments with the various ligands. When the absolute value of $\Delta F_{max}$ is around 2% or less, no accurate Ka value may be found. It can be inferred from Table 1 that a very low $\Delta F_{max}$ at one excitation wavelength does not necessarily mean that the ligand is not binding to the protein, as excitation at another wavelength sometimes gave a much high $\Delta F_{max}$, and therefore a measurable Ka. See for instance, the system E109 monomer-nystose in Table 1.

Monomeric immunoglobulins A4, A47N, E109 and U61 were run on agar versus 0.1% inulin. A4, A47N and U61 showed a single precipitin-line, but E109 did not precipitate. Methyl β-D-Fruf (0.4 M), methyl α-D-Glcp (0.4 M) and sucrose (0.2 M) were tested for their power to inhibit the three precipitating systems. Only methyl α-D-Glcp failed to inhibit precipitation.

Inulin is a linear polysaccharide containing β-D-Fruf units linked 2→1, and terminated, as in sucrose, by a similarly linked α-D-Glcp residue. Methylation studies by others have shown that the number average molecular weight of inulin is approximately 5000, involving about 28 intercatenary Fruf residues, one non-reducing terminal Fruf residues, and at the other terminal end a non-reducing Glcp residue.

We have assumed that one molecule of inulin binds to only one combining site of the immunoglobulins under the conditions of our titrations. From the work of Segal et al., here at NIH, on the structure of immunoglobulin Mc603 we know that the N-terminal portion of a Fab' fragment has a width of approximately 18 Å. It would thus appear possible that several immunoglobu-

TABLE 1

Maximum, ligand-induced changes in fluorescence (in %) of the myeloma proteins E109, A47N, A4 and U61, monitored at 340 nm after excitation at either 295 or 280 nm.

| Ligands | E109 | | | | A47N | | | |
|---|---|---|---|---|---|---|---|---|
| | monomer | | Fab' | | monomer | | Fab' | |
| | 295 | 280 | 295 | 280 | 295 | 280 | 295 | 280 |
| Me α-D-Glcp | -2.7 | --- | -0.8 | -0.7 | -2.1 | -0.6 | -1.2 | -1.2 |
| Me β-D-Fruf | -1.8 | -2.0 | -0.6 | -2.1 | -4.7 | -4.3 | -3.2 | -5.2 |
| Sucrose ($F_1G_1^1$) | -7.9 | --- | --- | --- | -4.2 | --- | --- | --- |
| 1-Kestose ($F_2G_1^1$) | -3.4 | --- | --- | -6.5 | -4.5 | --- | --- | -6.1 |
| Nystose ($F_3G_1^1$) | -2.1 | -6.5 | --- | -7.3 | -2.7 | -6.5 | --- | -6.6 |
| $F_4G_1^1$ | ∿0 | -6.6 | --- | -6.7 | <-1 | -4.8 | --- | -4.3 |
| Inulin ($F_nG_1^1$) | -3.7 | --- | -4.1 | --- | --- | --- | -5.7 | --- |

(Table 1 continued)

| Ligands | A4 | | | | U61 | | | |
|---|---|---|---|---|---|---|---|---|
| | monomer | | Fab' | | monomer | | Fab' | |
| | 295 | 280 | 295 | 280 | 295 | 280 | 295 | 280 |
| Me α-D-Glcp | -2.4 | -0.3 | -1.1 | -1.0 | -1.2 | ∿0 | -0.9 | -0.6 |
| Me β-D-Fruf | -1.8 | -2.2 | -2.6 | -6.3 | -0.5 | <-1 | -0.4 | -2.0 |
| Sucrose ($F_1G_1^1$) | -7.5 | --- | --- | --- | -4.5 | -7.2 | --- | -7.4 |
| 1-Kestose ($F_2G_1^1$) | -6.2 | --- | --- | --- | -1.4 | -2.7 | --- | --- |
| Nystose ($F_3G_1^1$) | -2.6 | -6.0 | -1.9 | -7.7 | <-1 | -2.5 | --- | -2.8 |
| $F_4G_1^1$ | --- | -7.5 | --- | -6.3 | --- | -3.9 | --- | -2.8 |
| Inulin ($FnG_1^1$) | --- | --- | -4.9 | --- | <-2 | -9.1 | --- | -5.2 |

residues may be involved in the ligand-induced altered fluorescence we have observed for these four proteins.

Table 1 lists the maximum changes (decreases) in fluorescence ($\Delta F_{max}$) for the monomeric myeloma IgA proteins A4, A47N, E109 and U61 as well as for their Fab' fragments, induced by the following ligands: methyl α-D-Glcp, methyl β-D-Fruf, sucrose ($F_1G_1^1$), 1-kestose ($F_2G_1^1$), nystose ($F_3G_1^1$) and the pentasaccharide $F_4G_1^1$. The $\Delta F_{max}$ for the four Fab' fragments in the presence of excess inulin is also given.

Ouchterlony double diffusion experiments showed that of the four monomeric immunoglobulins, only E109 failed to precipitate with inulin. Measurement of the $\Delta F_{max}$ values (and also the association constant) for any monomeric protein with inulin would obviously be fruitless if this is a precipitating system. A measurement of ΔF as a function of inulin added to an immunoglobulin is only meaningful in the case of E109. Indeed, the $\Delta F_{max}$ for this system (see Table 1) is only slightly lower than for the system E109 (Fab')-inulin.

Table 2 lists the association constants Ka for the four monomeric immunoglobulins and for their Fab' fragments with the various ligands. When the absolute value of $\Delta F_{max}$ is around 2% or less, no accurate Ka value may be found. It can be inferred from Table 1 that a very low $\Delta F_{max}$ at one excitation wavelength does not necessarily mean that the ligand is not binding to the protein, as excitation at another wavelength sometimes gave a much high $\Delta F_{max}$, and therefore a measurable Ka. See for instance, the system E109 monomer-nystose in Table 1.

Monomeric immunoglobulins A4, A47N, E109 and U61 were run on agar versus 0.1% inulin. A4, A47N and U61 showed a single precipitin-line, but E109 did not precipitate. Methyl β-D-Fruf (0.4 M), methyl α-D-Glcp (0.4 M) and sucrose (0.2 M) were tested for their power to inhibit the three precipitating systems. Only methyl α-D-Glcp failed to inhibit precipitation.

Inulin is a linear polysaccharide containing β-D-Fruf units linked 2→1, and terminated, as in sucrose, by a similarly linked α-D-Glcp residue. Methylation studies by others have shown that the number average molecular weight of inulin is approximately 5000, involving about 28 intercatenary Fruf residues, one non-reducing terminal Fruf residues, and at the other terminal end a non-reducing Glcp residue.

We have assumed that one molecule of inulin binds to only one combining site of the immunoglobulins under the conditions of our titrations. From the work of Segal et al., here at NIH, on the structure of immunoglobulin Mc603 we know that the N-terminal portion of a Fab' fragment has a width of approximately 18 Å. It would thus appear possible that several immunoglobu-

TABLE 2

Association constants, Ka ($M^{-1}$), for various ligands with the myeloma proteins E109, A47N, A4 and U61, determined by fluorescence titrations*.

| Ligands | E109 | | A47N | |
|---|---|---|---|---|
| | monomer | Fab' | monomer | Fab' |
| Me α-D-Glcp | $3.3(\pm0.2)\times10^2$ | ** | $1.5(\pm0.2)\times10^2$ | ** |
| Me β-D-Glcp | ** | ** | $1.7(\pm0.1)\times10^2$ | $1.35(\pm0.02)\times10^2$ |
| Sucrose ($F_1G_1^1$) | $0.93(\pm0.07)\times10^3$ | *** | $1.20(\pm0.03)\times10^3$ | *** |
| 1-Kestose ($F_2G_1^1$) | $0.97(\pm0.05)\times10^4$ | $1.2(\pm0.1)\times10^4$ | $0.71(\pm0.06)\times10^4$ | $1.04(\pm0.04)\times10^4$ |
| Nystose ($F_3G_1^1$) | $1.7(\pm0.1)\times10^5$ | $2.3(\pm0.1)\times10^5$ | $1.09(\pm0.02)\times10^5$ | $1.15(\pm0.02)\times10^5$ |
| $F_4G_1^1$ | $3.6(\pm0.2)\times10^5$ | $4.0(\pm0.2)\times10^5$ | $1.1(\pm0.1)\times10^5$ | $1.3(\pm0.1)\times10^5$ |
| Inulin ($F_nG_1^1$) | $11(\pm1)\times10^5$ | $6.6(\pm0.6)\times10^5$ | *** | $2.2(\pm0.2)\times10^5$ |

| Ligands | A4 | | U61 | |
|---|---|---|---|---|
| | monomer | Fab' | monomer | Fab' |
| Me α-D-Glcp | $1.4(\pm0.2)\times10^2$ | ** | ** | ** |
| Me β-D-Fruf | ** | $2.03(\pm0.06)\times10^2$ | ** | ** |
| Sucrose ($F_1G_1^1$) | $1.05(\pm0.05)\times10^3$ | *** | $0.68(\pm0.07)\times10^3$ | $0.53(\pm0.05)\times10^3$ |
| 1-Kestose ($F_2G_1^1$) | $1.10(\pm0.05)\times10^4$ | *** | $1.32(\pm0.07)\times10^4$ | *** |
| Nystose ($F_3G_1^1$) | $2.18(\pm0.03)\times10^5$ | $1.9(\pm0.2)\times10^5$ | $1.34(\pm0.04)\times10^5$ | $1.5(\pm0.1)\times10^5$ |
| $F_4G_1^1$ | $3.8(\pm0.2)\times10^5$ | $1.7(\pm0.1)\times10^5$ | $3.1(\pm0.1)\times10^5$ | $2.7(\pm0.2)\times10^5$ |
| Inulin ($F_nG_1^1$) | *** | $4.2(\pm0.3)\times10^5$ | *** | $3.1(\pm0.2)\times10^5$ |

*All titrations were done at that excitation wavelength giving the highest $\Delta F_{max}$ (Table 1).
**Ka could not be determined, $\Delta F_{max}$ too low.
***Ka not determined.



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lin combining sites could bind to inulin, since inulin in its extended form has a length of approximately 100 Å. However, in the case of the myeloma proteins studied here, the association constants of $F_4G_1^1$ with the anti-inulin Fab' fragments are essentially identical to those of inulin with the same fragments; the latter constants were calculated using the above mentioned assumption. Secondly, in the case of E109 and inulin, no precipitation occurred upon double diffusion. Thus, that preparation of E109 monomer is definitely incapable of binding more than one immunoglobulin combining site per molecule of inulin, otherwise crosslinking would occur, and precipitation take place. In the case of the other three immunoglobulins, the observed precipitation with inulin must therefore be attributed to non-covalent recombination of monomers to polymers in the purified immunoglobulin solution.

The apparent failure of E109 monomer to spontaneously recombine to dimers or polymers gave us the opportunity to accurately measure the affinity constant of an antigen and its whole immunoglobulin. Although there have been previous reports of affinity measurements between antigens and antibodies, to our knowledge this is the first such recorded instance using the fluorescence titration method. The constants of association obtained for inulin with E109 monomer and the corresponding Fab' fragment are very similar.

Methyl α-D-Glcp apparently binds to the E109, A47N and A4 monomers. The data on methyl α-D-Glcp indicate however that this simple glycoside binds to E109, A47N and A4 at a position other than the immunoglobulin combining site, perhaps somewhere on the Fc portion. Indeed, methyl α-D-Glcp was incapable of inhibiting the precipitation of A47N, A4 or U61 with inulin, thereby confirming that binding takes place outside the antibody combining site. In contrast, methyl α-D-Fruf seems to bind equally well with A47N monomer or its Fab' fragment.

The binding constants for sucrose ($F_1G_1^1$) to the four immunoglobulins studied, show that there nevertheless must be a small contribution of the glucose moiety to the binding of $F_1G_1^1$, as this ligand binds approximately five times better than methyl β-D-Fruf. Looking at the ligands, $F_1G_1^1$, $F_2G_1^1$, $F_3G_1^1$ and $F_4G_1^1$ shows that the binding affinities in this series reach an optimum value for $F_4G_1^1$, which is essentially identical with that of inulin with the same Fab' fragments. The immunodominant part of the oligosaccharide ligand appears to be the terminal Fruf unit, because methyl α-D-Glcp either binds very weakly or not at all. The size of the bindings site in these anti-inulin immunoglobulins is therefore thought to be approximately equal to the length of the tri- or tetra-Fruf segment in inulin, with maximum dimensions of approximately 14x14x4 Å (A47N, A4) or 15x14x10 Å (E109, U61). A hypothetical model of the Fv portion of E109, built by E. Padlan and M. Vrana (NIH) shows a combining site which agrees quite well with these dimensions (D.G. Streefkerk and C.P.J. Glaudemans).

TABLE 3

Maximum ligand-induced changes in fluorescence ($\Delta F_{max}$), association constants Ka (in $M^{-1}$), and binding energies ($\Delta G$, in cal.) for recombinant and native anti-inulin mouse myeloma immunoglobulins with inulin-related oligosaccharides.

| Ligand | | $H^{109}L^{61}$ | $H^{61}L^{109}$ | E109 | U61 | $H^{4}L^{957}$ | $H^{957}L^{4}$ | A4 | T957 | $H^{61}L^{61}$ |
|---|---|---|---|---|---|---|---|---|---|---|
| Sucrose | $\Delta F_{max}$ | -4.7% | -10.2% | -10.0% | -7.2% | -7.2% | -11.7% | -9.2% | -8.1% | -7.6% |
| $F_1G_1^1$ | Ka | $4.6\times10^2$ | $9.7\times10^2$ | $9.3\times10^2$ | $6.8\times10^2$ | $6.3\times10^2$ | $8.2\times10^2$ | $10.5\times10^2$ | $6.4\times10^2$ | $6.2\times10^2$ |
| | $\Delta G$ | 3650 | 4100 | 4050 | 3900 | 3850 | 4000 | 4150 | 3850 | 3800 |
| Nystose | $\Delta F_{max}$ | -2.5% | -5.1% | -6.5% | -2.5% | -4.2% | -7.5% | -6.0% | -5.9% | -3.4% |
| $F_3G_1^1$ | Ka | $0.84\times10^5$ | $1.90\times10^5$ | $1.73\times10^5$ | $1.34\times10^5$ | $1.5\times10^5$ | $2.3\times10^5$ | $2.18\times10^5$ | $2.06\times10^5$ | $0.84\times10^5$ |
| | $\Delta G$ | 6750 | 7200 | 7150 | 7000 | 7100 | 7350 | 7300 | 7250 | 6750 |
| | $\Delta F_{max}$ | -2.8% | -5.3% | -6.6% | -3.9% | -4.4% | -8.9% | -7.5% | -5.7% | -3.9% |
| $F_4G_1^1$ | Ka | $2.7\times10^5$ | $2.7\times10^5$ | $3.6\times10^5$ | $3.1\times10^5$ | $2.7\times10^5$ | $3.8\times10^5$ | $3.8\times10^5$ | $2.6\times10^5$ | $3.1\times10^5$ |
| | $\Delta G$ | 7400 | 7400 | 7600 | 7500 | 7450 | 7650 | 7650 | 7400 | 7500 |



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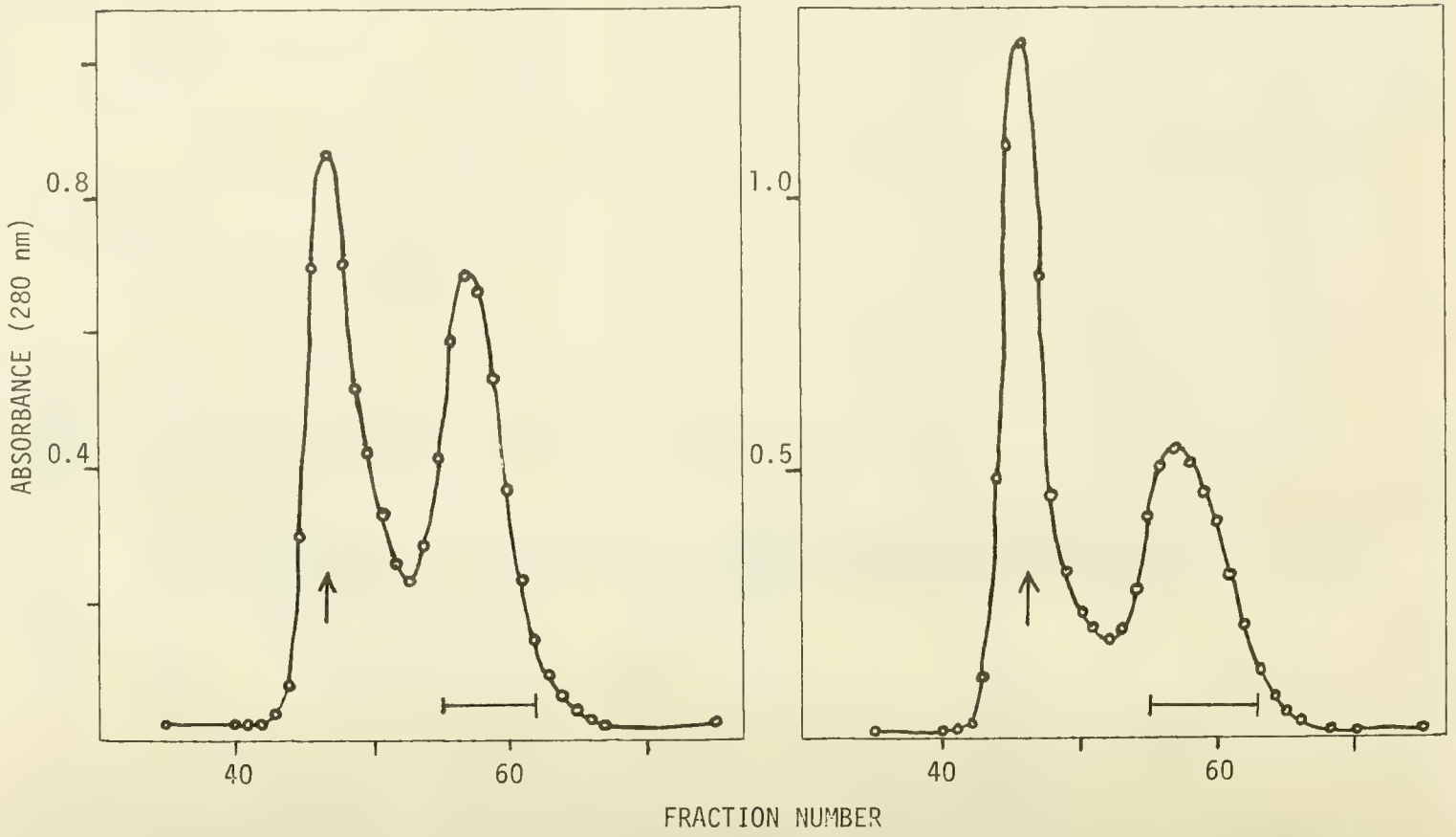
FIGURE 1

Fractionation of recombinants on Sephadex G200 (2.5x135 cm) in PBS, pH 7.4 (0.1% $NaN_3$). The void volume of the column, as determined by Blue Dextran, is indicated by the arrow. The second peak represents the 7S recombinant fraction. The horizontal bar indicates the fractions isolated for further analyses.



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In addition, similarly to the preparation of hybrid recombinants from immunoglobulins with anti-galactan specificity (see previous report), it became of great interest to continue this study by an evaluation of the binding behavior of hybrid recombinant immunoglobulins derived from the subunits (H and L chains) of a number of these anti-inulins.

It was generally found that the binding affinity of a given recombinant IgA for fructose oligosaccharides paralleled the binding affinity shown by the light-chain donor protein (D.G. Streefkerk and C.P.J. Glaudemans)(Table 3).

b. Anti-galactans.

Recombination of the heterologous H and L chains of S10 and J539 and subsequent chromatography on Sephadex G200 (Fig. 1), showed that the elution characteristics of these recombinant preparations were similar to our previously reported heterologous anti-galacatan recombinants.

The $H^{10}L^{539}$ and $H^{539}L^{10}$ 7S-recombinants thus isolated exhibited ligand-induced changes in fluorescence on binding galactose ligands. The constants of association (Ka) for some galactose-ligands with the 7S-recombinants $H^{10}L^{539}$ and $H^{539}L^{10}$ are given in Table 4, together with the maximally observed change in fluorescence on ligand binding. It was found that both recombinant immunoglobulins actively bound ligand showing an increase in tryptophanyl fluorescence with $Gal_2$ and $Gal_3$. However, the binding of MeGal to $H^{10}L^{539}$ caused a substantial decrease in the fluorescence of the protein. $H^{539}L^{10}$ showed a very small increase with the methyl glycoside. The reverse-sign ligand-induced fluorescence observed with the $H^{10}L^{539}$ recombinant is discussed later. The Ka values for $Gal_2$ and $Gal_3$ with $H^{10}L^{539}$, are close to those of S10 with these ligands, and the Ka values of $Gal_2$ and $Gal_3$ with $H^{539}L^{10}$ closely parallel those of J539 with these same ligands (Table 4). The Ka's of $H^{539}L^{10}$ and S10 with MeGal were not measured due to the low $\Delta F_{max}$ observed.

TABLE 4
Ligand binding characteristics of $H^{10}L^{539}$ and $H^{539}L^{10}$ 7S recombinants*

| Protein | Ka($M^{-1}$) | | |
|---|---|---|---|
| | MeGal | $Gal_2$ | $Gal_3$ |
| S10 | --- (-3.4%) | $0.49\times10^4$(+11.2%) | $0.28\times10^5$(+13.8%) |
| J539 | $1.01\times10^3$(+20%) | $1.25\times10^4$(+28%) | $1.5\times10^5$(+38.8%) |
| $H^{10}L^{539}$ | $0.76\times10^3$(-10.6%) | $0.7\times10^4$(+8.8%) | $0.38\times10^5$(+9.1%) |
| $H^{539}L^{10}$ | --- (+1.5%) | $1.1\times10^4$(+6.9%) | $1.5\times10^5$(+7.8%) |

*Bracketed values are the percentage changes in maximal fluorescence ($\Delta F_{max}$) upon binding the ligand in question with excitation and fluorescence radiation of 295 and 340 respectively.

The $\Delta F_{max}$ values observed with both the recombinants on binding $Gal_2$ and $Gal_3$ (Table 4) were very much lower than those previously observed with any of the other native or recombinant anti-galactans exhibiting ligand-induced changes in fluorescence (except for the previously reported value of 9% for $H^{10}L^{24}$). We had already found a case where the ligand-induced fluorescence change of a recombinant appears to depend mostly on the fluorescence change contributed by the H chain. If this holds true in the case studied here, it is not surprising that $H^{10}L^{539}$ shows a low value for $\Delta F_{max}$ in the case of $Gal_2$ and $Gal_3$, since S10 itself shows a small decrease in fluorescence on monosaccharide binding and only a moderate increase (11.2%) on disaccharide binding. However, J539 generally has a large $\Delta F_{max}$ on ligand binding (Table 4), and it would therefore be reasonable to expect a large $\Delta F_{max}$ for $H^{539}L^{10}$ and galactose-ligands. The observed value of 6.9% for that recombinant and $Gal_2$ suggested that the $H^{539}L^{10}$ 7S-recombinant may contain a considerable amount of denatured or inactive protein. This was proven to be the case.

It was found that $H^{539}$ in combination with $L^{10}$ shows a higher ligand-induced $\Delta F_{max}$ than does $L^{539}$ in combination with $H^{10}$. In addition the recombinant IgA having its H chain derived from the donor possessing reverse-sign fluorescence ($H^{10}L^{539}$) shows this phenomenon on ligand binding. Again, therefore, we have a second set of recombinants where the tryptophan residues in the heavy chain appear to determine the type of ligand-induced fluorescence changes in the heterologous recombinants.

The idiotypic specificity of the recombinants was analyzed by determining their capacity to inhibit the hemagglutination of myeloma protein-coated sheep erythrocytes by the corresponding idiotypic antisera. The results are listed in Table 5. If weak cross reactions are discounted, it can be seen that the antisera, except anti T601, are essentially individually specific. X24 was capable of inhibiting 601/anti-601 fairly well. This cross reactivity between the idiotypes of X24 and T601 has been noted before. Table 5 shows that the $H^{10}L^{539}$ and the $H^{539}L^{10}$ recombinants were idiotypically similar, but not identical, to the immunoglobulins donating the heavy chain.

3. Binding studies on anti-phosphorylcholine immunoglobulins from the mouse and anti-phosphorylcholine antibodies from the horse.

Eleven murine myeloma immunoglobulins with specificity for phosphorylcholine have been previously described, mostly by M. Potter's group at the NIH. The naturally occurring, "homologous" antigen for these immunoglobulins is not known, but it could be the species-specific C-teichoic acid (CTA) occurring in the cell walls of all Streptococci pneumoniae as shown by Brundis and Baddiley. Indeed, others have shown that immunization of mice with type R36a pneumococci (whose walls are a source rich in CTA) leads to an immune response, yielding an antibody-pool whose anti-PC antibodies consist nearly exclusively of immunoglobulins having the same idiotype as that

TABLE 5

Idiotypic specificity of recombinant molecules

| Inhibitor | $A_{280}$ | Hemagglutination inhibition titer (log 2) for the individual systems | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | J1/ anti-J1 | C4/ anti-C4 | S10/ anti-S10 | X24/ anti-X24 | X44/ anti-X44 | T191/ anti-T191 | J539/ anti-J539 | T601/ anti-T601 |
| J1 | 1.5 | 9 | 1 | 0 | 0 | 0 | 0 | 0 | 0 |
| C4 | 0.65 | 0 | 8 | 0 | 0 | 0 | 0 | 0 | 2 |
| S10 | 1.0 | 4 | 0 | 10 | 0 | 3 | 0 | 0 | 0 |
| X24 | 1.1 | 0 | 0 | 0 | 11 | 0 | 0 | 0 | 8 |
| X44 | 1.2 | 0 | 0 | 0 | 0 | 13 | 0 | 0 | 3 |
| T191 | 1.5 | 0 | 0 | 0 | 0 | 0 | 11 | 0 | 1 |
| J539 | 0.8 | 0 | 0 | 1 | 0 | 0 | 0 | 12+ | 0 |
| T601 | 2.5 | 0 | 0 | 0 | 0 | 0 | 0 | 2 | 8 |
| $H^{10}L^{539}$ | 1.1 | 0 | 0 | 6 | 0 | 0 | 0 | 0 | 0 |
| $H^{539}L^{10}$ | 1.5 | 0 | 0 | 3 | 0 | 0 | 0 | 8 | 0 |



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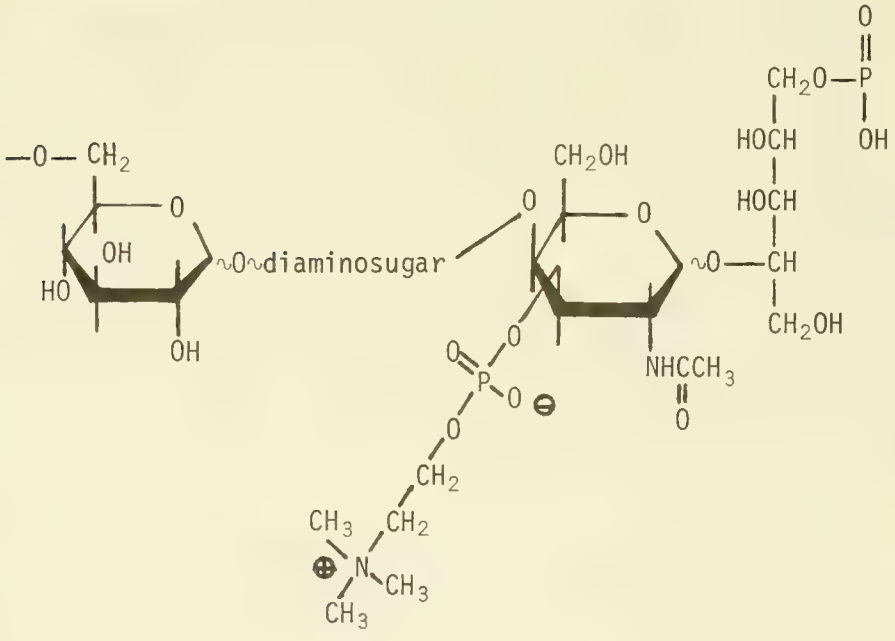
found on the IgA myeloma protein H8. CTA is known to be a polysaccharide whose repeating unit consists of glucose, a diamino dideoxyhexose, N-acetyl galactosamine and a ribitol phosphate, with phosphorylcholine (PC) attached to the galactosamine residue (Fig. 2).

FIGURE 2.




One member of the group of phosphorylcholine binding myeloma proteins, M603 has been crystallized and its three dimensional structure determined in Dr. Davies' group at NIH. It has been shown that this immunoglobulin possesses a large cavity in which PC is found to bind. This cavity is lined by the first, second, and third (H1, H2, and H3 respectively) hypervariable regions of the heavy chain and the first and third hypervariable region (L1 and L3) of the light chain. Furthermore, the interactions between the PC hapten and the immunoglobulin combining site are mediated predominantly through the heavy chains in M603, and the interactions are primarily electrostatic in nature. The high degree of sequence homology between the heavy chains of PC-binding proteins (S. Rudikoff and M. Potter at NCI) has permitted an assessment of the probable configuration of the hapten binding site in other proteins from this group. Although H8 has five amino acid moieties that differ from the corresponding ones in M603, it is still thought very likely that the combining cavities of H8 and M603 are very similar, since all the contact residues, which are involved in interaction betweer M603 and hapten occur in H8 as well.

When examining the combining site of M603 it can be observed that the hapten phosphorylcholine is relatively small compared to the large combining area. Since this hapten is located on the S. pneumoniae CTA antigen, it would be expected that an antibody combining site capable of binding this antigen must be large enough to allow at least part of the polysaccharide backbone to penetrate the site sufficiently to present the haptenic determinant to its particular binding environment. However, the question which arises is whether the remainder of the antigen binds in the combining site as well. Our approach to this problem has been to measure the binding affinities of whole antigen (CTA) with the immunoglobulin M603 and H8 Fab' fragments, and to compare this result with the affinity of these proteins for PC, the apparent haptenic portion of the antigen, and with S27, the capsular antigen from S. pneumoniae Type 27, whose repeating unit weight (908) is nearly identical to that of CTA (918) but is structurally unrelated to the latter antigen save for the presence of one PC moiety per repeating unit. The S27 repeating unit (established at NRC in Canada) consists of N-acetyl glucosamine (bearing a pyruvate), galactose, rhamnose (bearing PC), and glucose (Fig. 3).

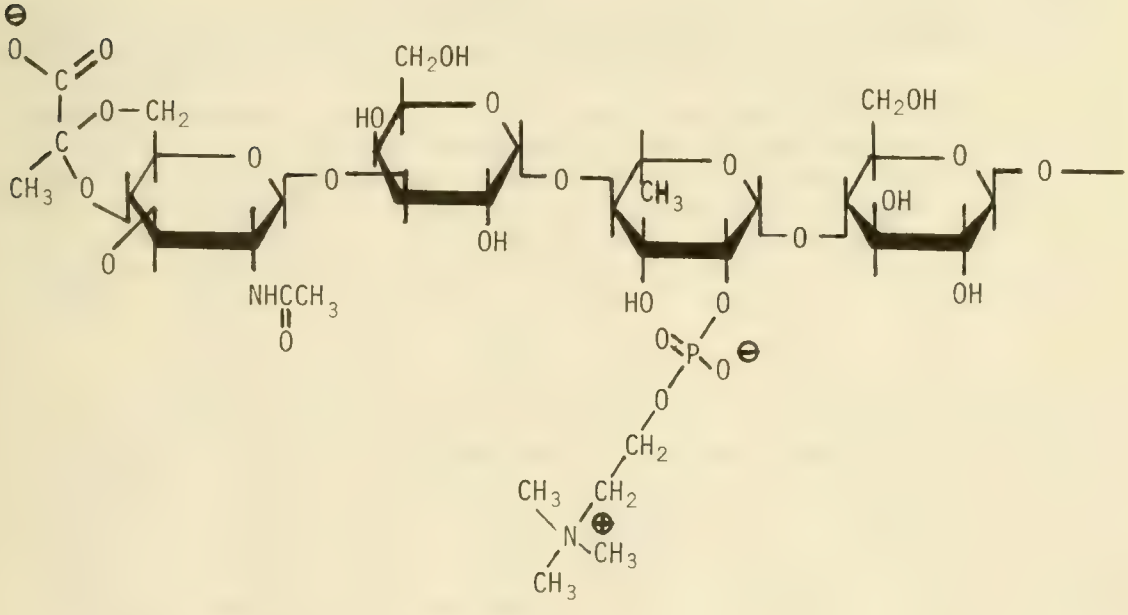


FIGURE 3.

The two antigens being studied, CTA and S27 have structurally nothing in common, save for PC and glucose, but the latter moiety is bound into the polysaccharide chains of CTA and S27 in different environments and by diffe ent linkages. Thus, binding of CTA and S27 to H8 and M603 is expected to be mediated only through their PC determinant. The similar association cor stants obtained with these two antigens and the lack of a substantial incre over the corresponding affinity constants of M603 and H8 with PC (Table 6) indicates that the polysaccharide backbone of either CTA or S27 neither bir strongly to the immunoglobulin in question, nor prevents the binding of the phosphorylcholine in the combining site. That the two antigens bind somewh more weakly than PC itself may be due to the fact that a polymeric antigen has fewer degrees of freedom and that diffusion (due to indefinite configur ational folding and unfolding) into an antibody combining-site thus will be diminished compared to that of a small ligand such as PC. CTA and S27 poly saccharide in all probability have no tertiary structure in solution since very few polysaccharides do. However, the fact that CTA has a straight-cha segment (ribitol) whereas S27 does not, may make the former antigen more flexible. This may explain the capability of CTA to bind IgA molecules in greater proximity to each other than does S27 in the precipitin reaction.

TABLE 6

The binding constants (Ka) of phosphorylcholine and two hosphorylcholin containing polysaccharides with murine myeloma immunoglobulins or their Fab' fragments.

| Antigen or Hapten | Immunoglobulin | Ka ($M^{-1}$) |
|---|---|---|
| Phosphorylcholine | H8 (Fab') | $4.6 \times 10^5$ |
| | H8 | $2.4 \times 10^5$ |
| | T15 (Fab') | $5.5 \times 10^5$ |
| | M603 | $1.6 \times 10^5$ |
| | M603 (Fab') | $1.7 \times 10^5$ |
| CTA (ATCC 12213) | H8 (Fab') | $4.0 \times 10^4$ $(0.8 \times 10^5)$* |
| | M603 (Fab') | $2.0 \times 10^4$ |
| S27 (S. pneumoniae) | H8 (Fab') | $1.8 \times 10^4$ $(0.5 \times 10^5)$* |
| | M603 (Fab') | $1.2 \times 10^4$ |

*Calculated by using 4.2 (for CTA) and 5.7 (for S27) repeating units of an gen per antibody combining site as determined by the precipiting curve for TEPC15 and these antigens.

Although immunization of mice with Streptococcus pneumoniae R36a yeilds immunoglobulins with the myeloma T15 or H8 site specific idiotype as part of the antibody pool, it is not possible to conclude that CTA is the natural or "homologous" antigen for some or all of the known homogeneous murine myeloma immunoglobulins. Potter and Lieberman (NCI) have suggested that phosphorylcholine-containing cell-wall components of gram-negative organisms may be the "homologous" antigen.

It is thus conceivable that another phosphorylcholine-containing macromolecular antigen could provide a better "fit" for these combining sites and thus produce a higher Ka as might have been expected rather than the slightly decreased Ka's observed with these antigens. Alternatively, the polysaccharide moieties may function merely as carriers and be irrelevant in terms of induction or specificity as suggested by the above data. It is possible that in the immunization experiments of Claflin and Davie (1974), the mice were capable of yielding part of their antibody pool in response to an immunogen (CTA in the walls of R36a cells) having only one relevant haptenic determinant (PC). It could be that the remainder of the antibody pool consists of antibodies whose binding sites show complimentarity for PC and carbohydrate moieties on the CTA. It is interesting that Dintzis and coworkers in 1976 indicated that the spacing of haptenic determinants in an antigen is an important factor in making this antigen immunogeneic. In any case, it appears that when an immunogen such as occurs in the cell wall of S. pneumoniae (CTA) is injected into Balb/c or A/J mice it is possible to obtain an immune response containing antibodies with the H8 idiotype and this idiotype is associated with the combining region of H8 (as worked out by Rudikoff and Claflin). Thus, it appears that an immunogen whose multivalent nature is due to the repetition of a single haptenic determinant can induce the formation of an antibody in animals if the haptenic determinant has a Ka of about $10^4$ with that antibody. Thus, it could be that the macromolecular backbone bearing this determinant need not contribute to the binding with the antibody in question.

It is interesting that phosphorylcholine, a fairly small molecule (about 9 Å), binds to H8 FAb' with an affinity constant of $4.6 \times 10^5$ $M^{-1}$. This is some forty times stronger than the binding of the larger galactodiose (about 13 Å) to the homogeneous immunoglobulin J539 Fab' (antigalactan specificity), previously reported by us. One difference between the nature of binding of these two ligands is that the phosphorylcholine and its antibody are involved in the interaction between six ions, two hydrogen bonds, and two sets of van der Waals interactions between $\pi$ and $\sigma$ electrons, whereas the galactodiose is a neutral molecule, and the nature of its interaction with the J539 combining site, although unknown, probably does not involve ionic bonding

In the horse there appears to be a different response: from horse antipneumococcal serum containing anti CTA, we isolated the PC binding antibody

pool. The affinity constants (Ka) of the Fab' fragments of this horse anti PC antibody pool, with PC, and the polysaccharides CTA and S27 are given in Table 7. It is apparent that the Ka for CTA is nearly 450 times higher than the one for PC, and that it is about 330 times higher than the one for S27.

TABLE 7

The affinity constants and the free energy for the interaction of PC and two PC containing polysaccharides with the Fab' of horse anti-pneumococcal antibody.

| Antigen or hapten | Ka($M^{-1}$) | ΔG(Kcal) |
|---|---|---|
| PC | $0.93 \times 10^4$ | -4.2 |
| S27 | $1.26 \times 10^4$ | -5.6 |
| CTA | $4.20 \times 10^6$ | -9.1 |

One can, therefore, expect that the binding of S27 to horse anti-pneumococcal CTA is mediated only through the PC determinant. Indeed, it can be seen from the data here that S27 and PC alone have approximately the same affinity for the antibody pool. However, the homologous antigen, CTA, has an affinity which is nearly 450 times stronger. We must conclude that this increased binding is mediated through the backbone polysaccharide of CTA. It is interesting to realize that the pool of antibodies studied were isolated with a PC-sepharose column and thus was selected for on the basis of its ability to bind to PC. Nevertheless, antibodies in this pool are capable of binding to the backbone polysaccharide, and thus display a type of affinity involving several determinants of the immunogen molecule. From the increase in free energy of binding between PC and CTA (5 kcal) it is clear that the additional affinity is quite strong.

These results with the horse anti-PC differ from our earlier results with the mouse myeloma anti-PC immunoglobulins, T15 and M603, wherein it was shown that the polysaccharide backbone of CTA does not contribute for the binding energy. Thus, while the immune system of mice recognize PC as the immunodominant moiety of the polysaccharide antigen, the immune system of horse can produce antibodies which can bind the polysaccharide moiety, in addition to the PC determinant (B.N. Manjula and C.P.J. Glaudemans).

4. Synthetic approaches to fluorescent ligands for immunoglobulins.

In connection with T-jump fluorescent studies it is sometimes necessary to employ fluorescent ligands. Several of these have been prepared by attaching dansyl moieties to various carbohydrates (E. Zissis and C.P.J. Glaudemans): 6'-trityl-gentiobiose heptaacetate when detritylated with HBr gave both anomers of gentiobiose heptaacetate. Treatment of either anomer with dansyl-chloride in pyridine gave two crystalline 6'-dansyl gentiobiose heptaacetates, and deacetylation then yielded crystalline 6'-dansyl gentiobiose, a fluorescent disaccharide derivative. Methyl β-D-galactopyranoside was directly dansylated at C6 and the crystalline acetate could be obtained from this. 6-O-β-D-6'-Dansyl-galactosyl-D-galactose could be prepared from the corresponding di-isopropylidene galactosyl-galactose by hydrolysis of the isopropylidene groups. These compounds are expected to be excellent fluorescent probes for anti-galactan antibodies.

Significance to Biomedical Research and the Program of the Institute

The Section on Carbohydrates, with its knowledge of immunochemistry and structural carbohydrate chemistry, is in a position to make a real contribution to the study of the immune response, employing these unique sets of immuno-globulins. The specificity of antibodies and the problem of the "ecomomy of the immune response" can be studied very well using this set of immunoglobulins.

Publications

Zissis, E. and Glaudemans, C.P.J.: The Anomers of p-Nitrophenyl 2,3,5-tri-O-benzyl-D-arabinofuranoside. Carbohyd. Res. 50: 292-295, 1976.

Tomasic, J.: Analysis of Gluronic Acid Conjugates. In Garrett, E.R. and Hirtz, J. (Ed.): Drug Fate and Metabolism: Methods and Techniques, Vol. 1. New York, Marcel Dekker, Inc., 1976.

Glaudemans, C.P.J., Manjula, B.N., Bennett, L.G. and Bishop, C.T.: The Binding of Phosphorylcholine-containing antigens from Streptococcus pneumoniae to Myeloma Immunoglobulins M603 and H8. Immunochem. 1977, in press.

Streefkerk, D.G. and Glaudemans, C.P.J.: Binding Studies on Anti-fructofuranan Mouse Myeloma Immunoglobulins A47N, A4, U61 and E109. Biochem. 1977, in press.

Glaudemans, C.P.J. and Jolley, M.E.: A method for Measuring Affinity between Carbohydrate Ligands and Certain Proteins. Methods in Carbohyd. Chem. VIII 1977, in press.

Glaudemans, C.P.J. and Bertolini, M.: O-chloroacetates of Sugars as Synthetic Intermediates. Methods in Carbohyd. Chem. VIII 1977, in press.

Glaudemans, C.P.J., Das. M.K. and Vrana, M.: Homogeneous Murine Immunoglobulins with Anti-carbohydrate Specificity. Methods in Enzymol. VIII 1977, in press.

Manjula, B.N., Mushinski, E.M. and Glaudemans, C.P.J.: The Formation of Active Hybrid Immunoglobulins from the Heavy and Light Chains of β(1,6) galactan Binding Murine Myeloma IgA's S10 and J539. J. of Immunol. 1977, in press.

| SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space) | U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT** | PROJECT NUMBER Z01 AM 19200-27 LC |
|---|---|---|

PERIOD COVERED

July 1, 1976 to September 30, 1977

TITLE OF PROJECT (80 characters or less)

Testing for Analgesic Activity, Toxicity, Dependence Liability and Narcotic Antagonism

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

| | | |
|---|---|---|
| PI: A. E. Jacobson | Research Chemist | NIAMDD-LC |
| Other: E. L. Atwell | Medical Biology Technician | NIAMDD-LC |

COOPERATING UNITS (if any)

Departments of Pharmacology, University of Michigan and Medical College of Virginia; Committee on Problems of Drug Dependence, Inc.

LAB/BRANCH

Laboratory of Chemistry

SECTION

Medicinal Chemistry

INSTITUTE AND LOCATION

NIAMDD, NIH, Bethesda, Maryland 20014

| TOTAL MANYEARS: | PROFESSIONAL: | OTHER: |
|---|---|---|
| 1.5 | 0.5 | 1.0 |

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

This project is designed for "base-line" studies for analgesic activity by the hot-plate and Nilsen methods (subcutaneous and oral administration) and acute toxicity for more interesting compounds. These more promising compounds are further assessed for physical dependence and antagonistic properties in Rhesus monkeys. Occasionally self-administration experiments (for psychologic abuse potential) are performed.



PHS-6040
(Rev. 10-76)

This program, in collaboration with the Committee on Problems of Drug Dependence, Inc., is to examine new compounds for analgesic activity (by the hot-plate and Nilsen methods), acute toxicity, properties of antagonism and abuse liability.

Approximately 143 new compounds from universities, the Laboratory of Chemistry and the (world-wide) pharmaceutical industry were submitted to this program and tested in the hot-plate and Nilsen methods, by the subcutaneous route of administration. Some of the more interesting compounds were tested for oral activity and for acute (24 hr.) toxicity. About 69 compounds were assessed for physical dependence liability and antagonistic property at the Departments of Pharmacology, University of Michigan and Medical College of Virginia. A few were examined for reenforcing (self-administration) properties (psychologic dependence potential).

The reports on thebaine are of especial interest. Thebaine is a rather toxic compound obtained from Papaver bracteatum, which has been considered as a possible replacement for Papaver somniferum (from which we presently obtain our supplies of morphine and, thus, codeine). We found that thebaine had definite analgesic activity (in the pethidine to codeine range) in rodent screens, and that it does not substitute for morphine in single dose suppression tests in monkeys. Its binding affinity to the opiate receptor *in vitro* was found to be $3 \times 10^{-6}$ nM. Due to the interest of the World Health Organization in this compound, and the disparate reports on its pharmacological activities, it will be studied more intensively.

Publications: None

| SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space) | U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT** | PROJECT NUMBER Z01 AM 19201-03 LC |
|---|---|---|

PERIOD COVERED

July 1, 1976 to September 30, 1977

TITLE OF PROJECT (80 characters or less)

Theoretical, In Vivo, and In Vitro Study of Analgesics and their Antagonists.

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

| | | | |
|---|---|---|---|
| PI: | A. E. Jacobson | Research Chemist | NIAMDD-LC |
| Other: | Jun-Ishi Minamikawa | Visiting Fellow | NIAMDD-LC |
| | M. F. Rahman | Visiting Associate | NIAMDD-LC |

COOPERATING UNITS (if any)

NIMH-LGCB (W. A. Klee, C. Pert, I. Kopin); University of Illinois at the Medical Center (W. J. Dunn); Medical College of Virginia (M. Aceto, L. Harris and E. L. May)

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SECTION

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NIAMD, NIH, Bethesda, Maryland 20014

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Different types of analgesics and narcotic antagonists were examined by a variety of in vivo and in vitro techniques to discern their effect on the narcotic receptor. Brominated narcotic antagonists were prepared, as were agonist-antagonists with unusual substituents. A theoretical study was completed to relate antinociceptive activity of diverse analgesics with their binding affinity to "opiate" receptors and their lipophilicity.



PHS-6040
(Rev. 10-76)

1. Brominated narcotic antagonists were prepared to explore routes to radiolabelled compounds for a collaborative study with Dr. I. Kopin (NIMH). The distribution of the compounds in normal mammalian brain will be studied to ascertain differences from the normal pattern which might occur during certain disease states.

Thus, 3-bromoallylnormorphine and 8-bromo-N-allylnormorphine were prepared. The latter was examined by Dr. C. Pert (NIMH) for *in vitro* binding affinity to the "opiate" receptor and was found to have binding affinity comparable to nalorphine. Our *in vivo* study showed it to have no appreciable analgesic activity and, thus, it would appear to be a suitable candidate for a strong narcotic antagonist.

*In vivo* and *in vitro* exploration of 3-bromoallylnormorphine will be continued.

2. In a theoretical study to see whether the antinociceptive activity of a diverse group of analgesics could be directly related to their binding affinity to "opiate" receptors, it was found that binding affinity alone was without predictive value. Regression analyses was employed to show that a linear combination of two independent parameters, binding affinity and lipophilicity, were sufficient for reasonable correlation to biological activity. For example, the antinociceptive activity of the potent analgesic fentanyl could not be predicted by a direct comparison of its binding affinity to "opiate" receptors ($1 \times 10^{-9}$) with that of morphine ($3 \times 10^{-9}$). An equation was generated so that a combination of the binding affinity and the lipophilicity of these molecules would, with reasonable accuracy, show that fentanyl was at least 200 times as potent as morphine *in vivo*.

This project has been completed.

3. In a project designed to examine the binding affinity of unusually substituted analgesics and their antagonists to the "opiate" receptor, several N-cyanoalkyl compounds were prepared. An N-cyanoethylmorphinan was found to be about 100 times as potent as morphine and appeared to act like a narcotic antagonist in single dose suppression studies in monkeys. It is, apparently, the only known narcotic antagonist in the morphinan series to have such extremely high analgesic activity.

These compounds will be further examined by withdrawal studies and in primary addiction studies in primate species.

Publications:

May, E. L. and Jacobson, A. E.: The chemistry and pharmacology of homologs of 6-acetyl and 3,6-diacetylmorphine. J. Pharm. Sci. 66: 285-286, 1977.

Yeh, H. J. C., Wilson, R. S., Klee, W. A. and Jacobson, A. E.: α- and β-Halomorphides: stereochemistry, analgesic potency, toxicity, and their interaction with narcotic receptors in vitro. J. Pharm. Sci. 65: 902-904, 1976.

Rice, K. C. Klee, W. A., Aceto, M. D., Swain, H. H. and Jacobson, A. E.: Potential long acting opiate antagonists: the preparation, pharmacological activity and opiate-receptor binding of N-substituted 2'-hydroxy-5-methyl-9α-propyl-6,7-benzomorphans. J. Pharm. Sci. 66: 193-197, 1977.

Jacobson, A. E.: Analgesics and their antagonists: structure-activity relationships. In Iversen, L. L., Iversen, S. D. and Snyder, S. H. (Eds.): Handbook of Psychopharmacology. New York, Plenum Publishing co., Inc. in press 1977.

Jacobson, A. E., Klee, W. A. and Dunn, W. J.: A quantitative relationship between antinociceptive activity, opiate receptor affinity and lipophilicity. Eur. J. Med. Chem. 12: 49-52, 1977.

Rice, K. C., Shiotani, S., Creveling, C. R. and Jacobson, A. E.: N-(2,4,5-trihydroxyphenethyl) normetazocine, a potential irreversible inhibitor of the narcotic receptor. J. Med. Chem., 673-675, 1977.

| SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space) | U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT** | PROJECT NUMBER ZO1 AM 19202-04 LC |
|---|---|---|

PERIOD COVERED

July 1, 1976 to September 30, 1977

TITLE OF PROJECT (80 characters or less)

Synthesis and Evaluation of Potential CNS, Antiinflammatory, and Anticancer Agents

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

| | | | |
|---|---|---|---|
| PI: | Kenner C. Rice | Research Chemist | NIAMDD-LC |
| Other: | A. E. Jacobson | Research Chemist | NIAMDD-LC |
| | M. Iorio | Visiting Scientist | NIAMDD-LC |
| | M. F. Rahman | Visiting Assocaite | NIAMDD-LC |

COOPERATING UNITS (if any) NIHM-LGCB (W. Klee); Medical College of Virginia (L. S. Harris, E. L. May, W. L. Dewey, M. E. Rogers, M. D. Aceto); Hoechst-Roussel Pharmaceuticals (W. Novick); Pennsylvania State University York Campus (E. A. Harrison)

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NIAMDD, NIH, Bethesda, Maryland 20014

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

A number of long acting narcotic antagonists were synthesized and evaluated in various in vivo and in vitro systems. Several potential stereospecific, irreversible binding agents to the narcotic receptor were also synthesized and evaluated. Several other types of potential CNS agents were also synthesized and examined in several in vivo and in vitro systems as well as a number of potential anticancer and antiinflammatory agents. Conditions were found for N-demethylation of morphine to normorphine under which no dihydronomorphine (a by-product formerly obtained in 5-10% yield) was produced.



PHS-6040
(Rev. 10-76)

The chemical synthesis and pharmacological evaluation of several 2'-hydroxy-6,7-benzomorphans and 3-hydroxymorphinans containing reactive functional groups was undertaken with the objective of obtaining stereospecific, irreversible binding agents to the narcotic receptor. Such an irreversible binding agent would be of great value in isolation and characterization of this protein which is thought to mediate analgesia and perhaps euphoria and certain other subjective effects of opiates. It was not possible to demonstrate stereospecific and irreversible binding for any of the compounds prepared. Further efforts along these lines are planned.

Pharmacological evaluation and opiate receptor binding of a series of 2'-hydroxy-N-alkyl-5-methyl-9α-propyl-6,7-benzomorphans was completed and our results indicate these compounds may be of value in drug rehabilitation programs as long acting narcotic antagonists (antidotes).

Several nitrogenous tetrahydrocannabinol-like compounds were prepared with the objective of gaining further insight into structural-activity relations in this area. These compounds were inactive as analgesics; screening for complete CNS profile is in progress.

The formation of dihydronormorphine as a by-product in the N-demethylation of morphine (by reaction of morphine with excess phenyl chloroformate and cleavage of the resulting carbamate with hydrazine) has been investigated and conditions have now been found which give 90% yield of the desired normorphine to the exclusion of the dihydro by-product.

A series of 3-thiosemicarbazones of 3-methyl-4-phenylpyridine-2-carboxaldehyde was prepared and evaluated for in vivo activity against P-388 leukemia in mice. Several of the compounds showed significant activity and are being considered for further evaluation by NCI.

Synthesis of several 2-aryl-3-hydroxy-thieno[2,3-b]quinolines as potential antiinflammatory compounds was carried out. Although these compounds were potent inhibitors of cyclic AMP phosphodiesterase in an in vitro system like a number of highly active antiinflammatory agents (inhibition of phosphodiesterase has been suggested as a rapid in vitro screen for in vivo antiinflammatory activity), these compounds were inactive in the carrageenan rat paw edema test for antiinflammatory activity. A number of interesting, novel chemical transformations of the thieno[2,3-b]quinoline system have been observed during synthesis of these compounds.

Based on results obtained earlier, a U.S. Patent application has been filed on synthesis of intermediates for Steroid total synthesis (Serial No. 690-361 filed May 26, 1976 with U. Weiss, NIAMDD, LPB).

Publications:

Rice, K. C. and Wilson, R. S.: (-)-3-Isothujone: A small non-nitrogenous molecule with antinociceptive activity in mice. J. Med. Chem. 19: 1054-1057, 1976.

Rice, K. C., Boone, B. J., Rubin A. B. and Rauls, T. J.: Synthesis, antimalarial activity and phototoxicity of some benzo[h]quinoline-4-methanols. J. Med. Chem. 19: 887-892, 1976.

Rice, K. C., Klee, Werner A. Aceto, M. D., Swain, H. H. and Jacobson, A. E.: Potential long acting opiate antagonists: The preparation, pharmacological activity and opiate-receptor binding of N-substituted 2'-hydroxy-5-methyl-9α-propyl-6,7-benzomorphans. J. Pharm. Sci. 66: 193-197, 1977.

Rice, K. C. and Wasylishen, R. E.: Carbon-13 NMR studies of some saturated 1,2-oxazepin derivatives. Org. Magn. Resonance 8: 449-452, 1976.

Rice, K. C., Shiotani, S., Creveling, C. R. and Jacobson, A. E.: N-(2,4,5-Trihydroxyphenethyl) normetazocine: A potential irreversible inhibitor of the narcotic receptor. J. Med. Chem. 20: 673-675, 1977.

Rice, K. C., Harrison, E. A., and Rogers, M. E.: Synthesis phosphodiesterase inhibition and antiinflammatory activity of 2-aryl-3-hydroxythieno[2,3-b]quinoline 1,1-dioxides. Application of sodium chlorite as a novel reagent for the stepwise oxidation of sulfides to sulfones. J. Heterocyclic Chem. in press 1977.

Rice, K. C. and Weiss, U.: Reaction of dimethyl 3-ketoglutarate with 1,2-dicarbonyl compounds. Simple synthesis of derivatives of 2,3,3a,4,5,9b-hexahydro-1H-benz[e]indene from dimethyl 3-keto-glutarate and glyoxal. J. Org. Chem. in press 1977.

Rice, K. C. and Weiss, U.: Intermediates for total steroid synthesis from the condensation product of dimethyl 3-ketoglutarate and glyoxal. U.S. Patent application pending (1977). Serial No. 690-361 filed May 26, 1976.

Rice, K. C. and May, E. L.: Procedural refinements in the N-demethylation of morphine and codeine using phenyl chloroformate and hydrazine. J. Heterocyclic Chem. in press 1977.

| SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space) | U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT** | PROJECT NUMBER Z01 AM 19206-1 LC |
|---|---|---|

PERIOD COVERED

July 1, 1976 to September 30, 1977

TITLE OF PROJECT (80 characters or less)

Nordihydromorphine, A By-product in the N-Demethylation of Morphine

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

| | | | |
|---|---|---|---|
| PI: | Everette L. May | Section Chief | NIAMDD-LC |
| Other: | Kenner C. Rice | Research Chemist | NIAMDD-LC |

COOPERATING UNITS (if any)

Section on Instrumentation and Analytical Services

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Medicinal Chemistry

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Final results reported in Z01 AM 19202-04



PHS-6040
(Rev. 10-76)

| SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space) | U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT** | PROJECT NUMBER Z01 AM 19208-02 LC |
|---|---|---|

PERIOD COVERED
July 1, 1977 to September 30, 1977

TITLE OF PROJECT (80 characters or less)
Synthesis and Pharmacology of (+)-Antipodes of Narcotic Agonists and Antagonists

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

| | | | |
|---|---|---|---|
| PI: | Kenner C. Rice | Research Chemist | NIAMDD-LC |
| Other: | I. Iijima | Visiting Scientist | NIAMDD-LC |
| | J. Minamikawa | Visiting Fellow | NIAMDD-LC |
| | A. Brossi | Visiting Scientist | NIAMDD-LC |

COOPERATING UNITS (if any)
NIMH (W. Klee); Medical College of Virginia (L. S. Harris, E. L. May, W. L. Dewey, M. D. Aceto); University of Michigan (H. H. Swain); New York State Research Institute for Neurochemistry and Drug Addiction (Y. Jacquet).

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NIAMDD, NIH, Bethesda, Maryland 20014

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

In order to provide research tools to study the phenomena of narcotic analgesia, addiction and tolerance, synthesis of the (+)-antipodes of codeine, morphine, heroin and naloxone was undertaken. (+)-Codeine and (+)-morphine have now been prepared in multigram amounts and subjected to detailed pharmacological evaluation. Synthesis of (+)-heroin and (+)-naloxone is nearing completion and pharmacological evaluation will follow.



PHS-6040
(Rev. 10-76)

Synthesis of the (+)-antipodes of codeine, morphine, heroin and naloxone was undertaken in order to provide sufficient quantities of these research tools to study the extent of the stereospecific effects of the (-)-isomers and to examine the question of multiplicity of morphine receptors with a view toward gaining further insight into the fundamental nature of narcotic addiction, analgesia and tolerance. A novel synthetic sequence has been developed and utilized for synthesis of multigram amounts of unnatural (+)-morphine and (+)-codeine from natural (-)-sinomenine (six steps, 25% overall yield). One key step in this sequence was the development of a rapid, high yield (90%) conversion of codeine to morphine, an efficient procedure for which had been lacking despite many attempts during the last 75 years. Pharmacological evaluation of (+)-morphine revealed that this substance was inert in a number of systems used to detect narcotic acticity but did produce dose dependent hyper-excitability and circling behavior in rats, quantatively similar to, but somewhat weaker than that characteristic of the (-)-isomer. Neither of these two effects produced by the two optical isomers was blocked or reversed by the narcotic antagonist (-)-naloxone. These and other data are suggestive of the existance of a morphine receptor with characteristics different from the morphine receptor presently recognized. Synthesis of (+)-heroin and (+)-naloxone (from (-)-sinomenine) is nearing completion and pharmacological evaluation of these compounds will begin upon completion of the synthetic work.

Publications:

Iijima, I. Rice, K. C. and Silverton, J. V.: Studies in the (+)-morphinan Series I. An alternate conversion of (+)-dihydrocodeinone into (+)-codeine. Heterocycles in press 1977.

Rice, K. C.: A rapid, high yield conversion of codeine to morphine. J. Med. Chem. 20: 164-165, 1977.

| SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space) | U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT** | PROJECT NUMBER Z01 AM 19209-01 LC |
|---|---|---|

PERIOD COVERED

July 1, 1976 to September 30, 1977

TITLE OF PROJECT (80 characters or less)

Unusual Heterocyclics via Physicochemical and Topological Analyses - Synthesis and Evaluation

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

| | | | |
|---|---|---|---|
| PI: | M. F. Rahman | Visiting Associate | NIAMDD-LC |
| Other: | K. C. Rice | Staff Fellow | NIAMDD-LC |

COOPERATING UNITS (if any)

Medical College of Virginia (M. E. Rogers); National Cancer Institute

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Medicinal Chemistry

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NIAMDD, NIH, Bethesda, Maryland 20014

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

This work has been discontinued.

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PERIOD COVERED

July 1, 1976 to September 30, 1977

TITLE OF PROJECT (80 characters or less)

Synthesis of Substituted Picolinamides.
2-Aryl-3-hydroxythieno [2,3-b]quinoline-1,1-dioxides.

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

| | | | |
|---|---|---|---|
| PI: | Ernest A. Harrison, Jr. | Research Chemist | NIAMDD-LC |
| Other: | Kenner C. Rice | Research Chemist | NIAMDD-LC |

COOPERATING UNITS (if any)

Medical College of Virginia (M. Rogers)

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NIAMDD, NIH, Bethesda, Maryland 20014

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| 0.3 | 0.3 | 0 |

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Synthesis of Substituted Picolinamides has been temporarily discontinued. 2-Aryl-3-hydroxythieno [2,3-b]quinoline-1,1-dioxides has been continued on #19202-04



PHS-6040
(Rev. 10-76)

| SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space) | U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT** | PROJECT NUMBER Z01 AM 19211-01 LC |
|---|---|---|

PERIOD COVERED

July 1, 1976 to September 30, 1977

TITLE OF PROJECT (80 characters or less)

The Synthesis and Pharmacological Examination of Retinoids

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

| | | |
|---|---|---|
| PI: A. E. Jacobson | Research Chemist | NIAMDD-LC |
| Other: A. Brossi | Chief, Medicinal Chemistry | NIAMDD-LC |

COOPERATING UNITS (if any)

NCI (M. Sporn)

LAB/BRANCH

Laboratory of Chemistry

SECTION

Medicinal Chemistry

INSTITUTE AND LOCATION

NIAMDD, NIH, Bethesda, Maryland 20014

| TOTAL MANYEARS: | PROFESSIONAL: | OTHER: |
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☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Retinal (Vitamin A aldehyde) and C-13 cis retinal have been utilized as starting materials to prepare compounds with improved organ and tissue distribution in vivo, for the inhibition of carcinogenic processes.



PHS-6040
(Rev. 10-76)

Retinylideneacetone, retinylideneacetylacetone and C-13 cis-retinylideneacetone were prepared in sufficient quantity to examine their effect in the inhibition of cancer in vitro, and in vivo in animal screens (in collaboration with M. Sporn, NCI).

Retinal and its unsaturated chain-elongated derivatives are known to inhibit the incidence and number of induced mammary cancers, et al. One problem with the utilization of these compounds for these purposes has been its distribution and storage in liver and consequent toxicity. Thus, related retinoids already reported in the literature in connection with Vitamin A chemistry will be synthesized and examined for improved distribution characteristics.

Publications: None

| SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space) | U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT** | PROJECT NUMBER Z01 AM 19212-01 LC |
|---|---|---|

PERIOD COVERED

July 1, 1976 to September 30, 1977

TITLE OF PROJECT (80 characters or less)

Practical Total Synthesis of Racemic Codeine and Other Important Opium Alkaloids

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT


| | | | |
|---|---|---|---|
| PI: | Kenner C. Rice | Research Chemist | NIAMDD-LC |
| Other: | A. Brossi | Chief, Medicinal Chemistry | NIAMDD-LC |
| | M. F. Rahman | Visiting Associate | NIAMDD-LC |


COOPERATING UNITS (if any)

University of Wisconsin (D. Pearlman); University of Minnesota (L. Miller)

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☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)


A vastly improved synthetic method which does not require protecting groups and which is readily applicable to large scale work has been extended to synthesis of a number of N-acyl norreticuline derivatives which are important precursors of opium alkaloids. Preliminary phenolic oxidation experiments have given racemic N-acyl salutaridine dervatives which can be readily converted to racemic codeine and other opium alkaloids using established methods.




PHS-6040
(Rev. 10-76)

This project was initiated in order to provide a continous source of unnatural (+)-codeine and (+)-morphine required for antiinflammatory and other pharmacological testing as well as an alternate (to the opium poppy) source of the medically valuable (natural) (-)-codeine. The approach employed in this work is along the biosynthetic lines, that is, oxidative coupling of a reticuline derivative to a salutaridine derivative. We have extended a superior synthetic sequence which is readily adaptable to a commercial scale, to the synthesis of N-acyl reticuline derivatives, formerly only available by complex multistep synthetic sequences involving introduction and removal of benzyloxy protecting groups. The synthetic sequence utilized by us does not require protecting groups and readily affords the required reticuline derivatives and with slight modification the corresponding 2'-bromo derivatives. Preliminary phenolic oxidation experiments have yielded solutaridine derivatives which can be converted to codeine and morphine derivatives using existing methods. Further experiments aimed at improving the yield in this key oxidative coupling step are in progress.

Publications: None

| SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space) | U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT** | PROJECT NUMBER Z01 AM 19213-01 LC |
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PERIOD COVERED
July 1, 1976 to September 30, 1977

TITLE OF PROJECT (80 characters or less)

3,4-Disubstituted Morphinanes

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

| | | | |
|---|---|---|---|
| PI: | A. Brossi | Chief, Medicinal Chemistry | NIAMDD-LC |
| Other: | M. F. Rahman | Visiting Associate | NIAMDD-LC |

COOPERATING UNITS (if any)

None

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INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20014

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Structure-activity studies within a series of 3,4-substituted morphinanes of the (-)-series have shown that 3,4-dimethoxy-N-methyl morphinan is an interesting analgesic.



PHS-6040
(Rev. 10-76)

Only few morphinanes disubstituted in the aromatic ring have systematically been explored. Since (-)-dihydrothebainone can easily be prepared from natural thebaine we have converted it by standard procedures into a variety of 3,4-disubstituted N-methyl morphinanes. Among those prepared (-)-3,4-dimethoxy-N-methyl morphinane showed an unexpected high activty, being almost equally active as levorphanol in the hot-plate assay.

Publication:

Rahman, M. F. and Brossi, A.: Preparation and analgesic activity of some 3,4-disubstituted N-methylmorphinanes of the (-)-series. Heterocycles (Japan) in press 1977.

| SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space) | U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT** | PROJECT NUMBER Z01 AM 19214-01 LC |
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PERIOD COVERED

July 1, 1976 to September 30, 1977

TITLE OF PROJECT (80 characters or less)

Direct and Sequential Removal of Phenolic Oxygen Functions in Alkaloids

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

| | | | |
|---|---|---|---|
| PI: | A. Brossi | Chief, Medicinal Chemistry | NIAMDD-LC |
| Other: | K. C. Rice | Research Chemist | NIAMDD-LC |
| | M. F. Rahman | Visiting Associate | NIAMDD-LC |

COOPERATING UNITS (if any)

Department of Pharmacology, University of Toronto, Canada ( P. Seeman)

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| 0.5 | 0.5 | 0 |

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Phenolic groups in alkaloids can be removed by cleavage of their phosphate esters with alkali metals in liquid ammonia or by catalytic hydrogenation of their tetrazolyl ethers. Direct and sequential removal of present or newly created phenolic substituents in the series of aporphine alkaloids could be successfully completed. This work started originally at the Hoffmann-La Roche Laboratories in Nutley, New Jersey.



PHS-6040
(Rev. 10-76)

In collaboration with Drs. Teitel and Gerecke from the Roche Laboratories at Nutley, N. J. (USA) and Basle, Switzerland the three aporphines S-(+)-bulbocapnine, S-(+)-boldine and R-(-)-apomorphine have been converted by ether cleavage and removal of the phenolic oxygen function, mimicked as phosphate ester or tetrazolyl ether with lithium in liquid ammonia at -78° or catalytic hydrogenation over 10% Pd-C in AcOH at room temperature, respectively, into a variety of less oxygenated congeners. The apomorphines obtained are being evaluated as dopaminergic agents in Prof. Seeman's laboratory.

The generality of this method is presently being evaluated in other classes of alkaloids. It has now been shown that removal of the phenolic group in dihydromorphine gives the highly potent analgesic desoxy dihydromorphine. The high activity of this compound lacking the original phenolic function is surprising.

Publications:

Brossi, A., Rahman, M. F., Rice, K. C., Gerecke, M., Borer, R., Teitel, S. and O'Brien, J. P.: Direct and sequential removal of oxygen functions in S-(+)-bulbocapnine, (S)-boldine and R-(-)-apomorphine. Heterocycles (Japan) Woodward issue 1977.

| SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space) | U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT** | PROJECT NUMBER Z01 AM 19215-01 LC |
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PERIOD COVERED

July 1, 1976 to September 30, 1977

TITLE OF PROJECT (80 characters or less)

Condensation of Biogenic Amines with Carbonyl Compounds under "So-called" Physiological Conditions

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

| | | | |
|---|---|---|---|
| PI: | A. Brossi | Chief, Medicinal Chemistry | NIAMDD-LC |
| Other: | M. Iorio | Visiting Scientist | NIAMDD-LC |
| | A. E. Jacobson | Research Chemist | NIAMDD-LC |

COOPERATING UNITS (if any)

NCI-D (J. H. Robbins); Department of Neurology, Mount Sinai School of Medicine, N. Y. (G. Cohen).

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Formaldehyde and acetaldehyde were the only carbonyl compounds which reacted with histamine, dopamine and serotonine in a buffered solution of pH 7.4 at 37°. No reaction could be observed with tryptamine and 6-hydroxy-dopamine.



PHS-6040
(Rev. 10-76)

Most of the reported *in vitro* reactions between biogenic amines and carbonyl compounds, a reaction which is suggested to take place *in vivo*, have been carried out under acidic conditions. In order to evaluate the hypothesis that such reactions might occur in the human brain, *in vitro* conditions had to be found which would mimick the *in vivo* situation. The biogenic amines dopamine, 6-hydroxy dopamine, tryptamine, serotonine and histamine were allowed to react with formaldehyde, acetaldehyde, acetone and 5-amino-levulinic acid in a phosphate buffered solution of pH 6.6-7.4 for 24 hours at 37° in dilute concentration ($2 \times 10^{-2}$ M). Formaldehyde and acetaldehyde were the only carbonyl compounds which gave visible reactions with histamine, serotonine and dopamine (color change, tlc). Dopamine and 6-hydroxydopamine are very unstable under these reaction conditions and are converted into highly colored materials.

Publications: None

| SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space) | U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT** | PROJECT NUMBER Z01 AM 19216-01 LC |
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PERIOD COVERED

July 1, 1976 to September 30, 1977

TITLE OF PROJECT (80 characters or less)

Molecular Features of Colchicine Associated with Antimitotic and Antigout Activity

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

| | | | |
|---|---|---|---|
| PI: | A. Brossi | Chief, Medicinal Chemistry | NIAMDD-LC |
| Other: | M. Iorio | Visiting Scientist | NIAMDD-LC |

COOPERATING UNITS (if any)

NIAMDD-LEP (J. A. Sipe); NCI-I (K. P.W.J. McAdam); NCI-DR&D (H. B. Wood, Jr.)

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NIAMDD, NIH, Bethesda, Maryland 20014

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☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Natural (-)-colchicine can easily be converted into trimethyl colchicinic acid (TMCA). The latter can be racemized in the form of its Schiff's base with KOH in methanol. Mescaline has been converted by standard procedures into a variety of N-trimethoxybenzyl and 1-trimethoxyphenyl substituted tetrahydroisoquinolines to be evaluated in vitro as antimitotic agents



PHS-6040
(Rev. 10-76)

The antimitotic activity of colchicine is the result of a 1:1 interaction between colchicine and tubulin, a dimeric protein which aggregates to form the microtubules of the mitotic spindle. Among a series of colchicine analogs, a good correlation seems to exist between antimitotic activity and ability to bind to the colchicine-binding site of tubulin. Fitzgerald's finding that a substance must contain two aromatic rings substituted with methoxy groups, as present in desacetamidocolchicine, methoxy substituted phenyl-tropones and biphenyls, is rather surprising, since natural products exhibiting specific biological activities are usually much more demanding with regard to structural and topographical features. We are now in the process to prepare larger quantities of unnatural (+)-colchicine, prepared earlier by Corrodi and Hardegger on an academic scale, to test this hypothesis.

In our hand colchicine can easily be converted into crystalline trimethyl colchicinic acid (TMCA). Its Schiff's base with benzaldehyde can be racemized with methanolic KOH in high yield to afford directly crystalline racemic TMCA. The latter constitutes a vital intermediate in our studies.

From mescaline, another compound which has been reported to have antimitotic properties, several tetrahydroisoquinolines were prepared by the following routes:

A. Condensation with 2,4,5- and 3,4,5-trimethoxy-benzaldehyde, reduction of the Schiff's bases with borohydride and condensation of the secondary amines thus obtained with formaldehyde in the presence of acid.

B. Preparation of the amide from mescaline and 3,4,5-trimethoxy benzoic acid, cyclization with oxychloride, reduction followed by N-methylation or aromatization.

Publications: None

| SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space) | U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT** | PROJECT NUMBER Z01 AM 19400-12 LC |
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PERIOD COVERED

July 1, 1976 through September 30, 1977

TITLE OF PROJECT (80 characters or less)

Pharmacologically Active Compounds from Tropical Frogs and Other Natural Sources

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: John W. Daly, Chief, Section on Pharmacodynamics, NIAMDD LC
Bernhard Witkop, Chief, Laboratory of Chemistry, NIAMDD LC

Other: C. R. Creveling, Research Chemist, NIAMDD LC
Wolfgang Burgermeister, Visiting Associate, NIAMDD LC
Alain Van Dorsselaer, Visiting Fellow, NIAMDD LC

COOPERATING UNITS (if any) C. W. Myers, Am. Museum of Nat. History, N.Y.C.; E. X. Albuquerque, M. E. and A. T. Eldefrawi, Univ. Md. School Medicine, Baltimore, MD.; I. L. Karle, U. S. Naval Res. Lab., Wash., D. C.; G. Habermehl, Technische Hochschule, Darmstadt, Germany; T. Tokuyama, Osaka City Univ., Osaka, Japan; E. Bartels

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SECTION
Section on Pharmacodynamics

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland, 20014

| TOTAL MANYEARS: | PROFESSIONAL: | OTHER: |
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| 1 | 0.6 | 0.4 |

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☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

A variety of diverse pharmacologically active alkaloids have been isolated from skin extracts of dendrobatid frogs and their structures and pharmacology investigated. These include gephyrotoxin, a novel tricyclic alkaloid with marked activity as a muscarinic antagonist; pumiliotoxin A and B, complex alkaloids with pronounced cardiotonic activity; and finally an alkaloid of unknown structure with potent analgesic activity. Radioactive perhydrohistrionicotoxin did not bind at the acetylcholine receptor purified from the electroplax of the electric ray, but did bind with high affinity to an associated but separable ion conductance modulator. Interaction of batrachotoxin with the sodium channel in innervated cells of the electroplax of electric eel required membrane depolarization. Dihydroisohistrionicotoxin altered binding of agonists and antagonists to acetylcholine receptors in cultured neuroma cells and cultured skeletal muscle cells. In muscle cells the toxin appears to bind to an allosteric effector site, thereby desensitizing the channel and altering the acetylcholine-receptor site.



PHS-6040
(Rev. 10-76)

Cooperating Units (Cont'd.): de Bernal, Universidad de Valle, Cali, Colombia; T. Rosenberry, Columbia University, N.Y.C.; L. Tice, Laboratory of Experimental Pathology, NIAMDD; Y. Kishi, Harvard University, Cambridge, Mass., M. Nirenberg, Lab. Biochemical Genetics, NHLI; W. A. Catterall, Lab. Biochemical Genetics, NHLI.

Project Description:

Objectives: To elucidate the molecular basis for the pharmacological activity of batrachotoxin, tetrodotoxin, histrionicotoxin, pumiliotoxin, and related substances. To isolate and elucidate the structures of other pharmacodynamically active substances found in skin extracts of frogs and other natural sources. To explore biosynthetic pathways involved in the formation of these unique substances.

Methods Employed: Sensitive bioassay methods, thin layer, high pressure liquid, column, and gas chromatography have been used in the purification of the active principles, while NMR, mass spectroscopy, X-ray crystallography, microchemical techniques and radiochemical labeling are being utilized for structure elucidation. Isotopic tracer techniques have been used to study biosynthethic pathways. Biological activity has been investigated with both isolated enzyme systems, in intact cells and muscle preparations and in vivo.

Major Findings: Further quantities of dendrobatid alkaloids have been isolated for determination of structure and investigation of their unique pharmacological properties. Gephyrotoxin, an active muscarinic antagonist was shown to be a unique tricyclic alkaloid by X-ray crystallography. In toto, nearly 100 different alkaloids have been detected and characterized in extracts from over thirty different species of dendrobatid frogs. These alkaloids have been divided into six different classes and structures assigned to the majority. A 2,6-disubstituted piperidine has been detected in one species and perhaps represents a precursor class for the biosynthesis of the bicyclic histrionicotoxins and pumiliotoxins. Certain disubstituted piperidines were synthesized for pharmacological investigation. The dendrobatid alkaloids are proving invaluable to the definition of intraspecific, intrageneric, evolutionary, and ecological relationships in this family of frogs. As yet they have not been detected in any other family of frogs. A small neotropical toad of the genus Dendrophryniscus was found to contain unique lipid soluble neutral compound with long-lasting sedative activity (Daly).

A water soluble toxin was found in significant amounts in roe and liver from a fish of the Red Sea. The properties of this toxin are very much like those of tetrodotoxin. Extracts of engorged ticks from Colorado contained only very low levels of a paralytic toxin (Daly).

Batrachotoxin did not cause depolarization of cells in the innervated membrane of the electroplax of the electric eel. If the membrane was partially depolarized by electrical stimulation, carbamylcholine, or high concentrations of potassium, batrachotoxin caused a complete and relatively irreversible depolarization. The results indicate that the interaction of batrachotoxin with action potential sodium channels is strongly voltage dependent (Daly).

Radioactive perhydrohistrionicotoxin bound reversibly and with high affinity to a limited number of sites in membranes from the electroplax of the Torpedo fish. After solubilization the perhydrohistrionicotoxin-binding site could be separated from the acetylcholine receptor. The data were, in toto, consonant with the presence of perhydrohistrionicotoxin-binding sites on the ion conductance modulator of the acetylcholine receptor. The modulator and receptor appear to be readily dissociable entities. Interaction of histrionicotoxins with this site on the modulator is responsible for blockade of responses to acetylcholine. (Daly, Witkop).

Dihydroisohistrionicotoxin noncompetitively inhibited binding of scopolamine to muscarinic receptors in neuroblastoma cells and neuroblastoma-glioma hybrid cells. Tetracaine had similar effects on the binding of scopolamine (Burgermeister).

Cultured chick-embryo skeletal muscle cells possess a high concentration of nicotinic acetylcholine (ACh) receptors. Stimulation of these receptors with 1 mM carbamylcholine greatly increases the ion permeability of the cells. The cultured cells have provided a valuable system for studying the effects of dihydroisohistrionicotoxin (DHTX) on (i) the sodium transport activity of the ACh receptor, and (ii) the affinity of the ACh receptor for cholinergic ligands. DHTX inhibits the ACh receptor-mediated uptake of sodium into the cells with an $IC_{50}$ of 0.5 μM. Binding of DHTX to the ACh receptor occurs only at a much higher concentration of the toxin as was demonstrated by its ability to inhibit binding of [$^{125}$I]α-bungarotoxin to the receptor ($IC_{50}$ = 30 μM). Further studies have shown that DHTX has an allosteric effect on the ACh recognition site. Binding of the ACh receptor agonists, carbamylcholine, ACh, decamethonium, nicotine, and the ACh receptor antagonists, d-tubocurarine, and gallamine, was measured by inhibition of [$^{125}$I]α-bungarotoxin binding to the ACh receptor of the cells. In the presence of a very low concentration of DHTX binding of the agonists was increased whereas binding of the antagonists was unchanged. The $IC_{50}$ values of the agonists were reduced to 1/10 to 1/20 of the $IC_{50}$ values observed in absence of DHTX. By measuring the initial rate of α-bungarotoxin binding in absence and presence of carbamylcholine and DHTX, it was found that the change of receptor affinity for carbamylcholine occurs within the first 5 minutes of incubation. Affinity changes of equal size and rate can be produced by either 5 μM carbamylcholine plus 1 μM DHTX or 40 μM carbamylcholine without DHTX. When the cells are preincubated with 40 μM carbamylcholine and then stimulated with 1 mM carbamylcholine, the ACh receptor-mediated sodium transport is progressively reduced. This phenomenon, which is caused by desensitization of the ACh receptor, shows a very similar time dependence as the receptor affinity change for carbamylcholine produced by either 40 μM carbamylcholine or 5 μM carbamylcholine plus 1 μM DHTX. The following hypothesis is proposed for the action of DHTX. The toxin binds to a site allosteric to the ACh recognition site of the ACh receptor. Thereby a conformational transition of the ACh receptor is caused. The resulting ACh receptor conformation represents a desensitized state since it does not mediate sodium transport. It differs from the resting state of the ACh receptor by having a higher affinity for receptor agonists (Burgermeister).

Derivatives of pseudo-pelletierine (N-methyl-granatanone) which have structural similarities with histrionicotoxin have been synthesized by N- and

C-alkylations. N-pentyl and N-benzyl derivatives of pseudo-pelletierine and the two corresponding alcohols of each (3α- and 3β-) have been synthesized. The addition of n-pentyl to the carbonyl group was impossible with the Grignard reagent because of the low reactivity of pseudo-pelletierine, but the analogous reaction with lithium and n-bromopentane was successful. Alkylation of N-pentyl and N-benzyl derivatives of pseudo-pelletierine should yield the di-alkylated pseudo-pelletierines. Preliminary results indicate that $5 \times 10^{-5}$ M N-methyl,3-pentyl-granatan-3β-ol, like histrionicotoxin and like dihydroadaline (a related alkylated pseudo-pelletierine), inhibits sodium uptake elicited by carbachol by 50% in chick muscle cells (Van Dorsselaer, Burgermeister).

Significance to Biomedical Research and the Program of the Institute: The exceptionally high toxicity of batrachotoxin, its selective action on nerve preparations and its cardiotoxin properties gave the elucidation of its structure and correlations of structures with activity a high priority in biomedical research. Because of their remarkable effects on ion conductances, histrionicotoxin and various analogs provide unique tools for the study of the acetylcholine-modulated ion channels in electrogenic membrane. Gephryotoxin provides a new class of muscarinic antagonists. Other compounds from frog, fish, insect, and plant extracts also have physiological activities which warrant their structural and pharmacological investigation. Examples of such compounds occurring in frogs and toads include the pumiliotoxins, samandarine, the bufogenins, the catecholamines, indolealkylamines and histamines, and hypotensive polypeptides, such as bradykinin and physalaemin. The potent analgesic and cardiotonic effects of certain of the alkaloids are of particular relevance to the design of new drugs.

Proposed Course of Project: The structures of further alkaloids from frogs of the genera Dendrobates, Colosthetus, and Phyllobates will be elucidated. The pharmacological activity of batrachotoxin, pumiliotoxin, and histrionicotoxin analogs will be studied in further detail. Radioactive batrachotoxin, tetrodotoxin, and histrionicotoxin will be used to study their ultrastructural site of action. Further isolations of batrachotoxins, histrionicotoxins, pumiliotoxins, and related alkaloids will be carried out to provide material for chemical and pharmacological study. Pharmacologically active substances will be investigated in extracts from other families of tropical frogs, from fish, insects, and plants. Further synthetic analogs of dendrobatid alkaloids will be prepared.

Publications:

Bartels-Bernal, E. Rosenberry, T. L., and Daly, J. W.: Effect of batrachotoxin on the electroplax of electric eel: Evidence for voltage-dependent interaction with sodium channels. Proc. Natl. Acad. Sci. USA. 74: 951-955, 1977.

Daly, J. W., Witkop, B., Tokuyama, T., Nishikawa, T., and Karle, I. L.: Gephyrotoxins, histrionicotoxins, and pumiliotoxins from the neotropical frog Dendrobates histrionicus. Helv. Chim. Acta 60: 1128-1140, 1977.

Eldefrawi, A. T., Eldefrawi, M. E., Albuquerque, E. X., Oliveira, A. C., Mansour, N., Adler, M., Daly, J. W., Brown, G. B., Burgermeister, W., and Witkop,

B.: Perhydrohistrionicotoxin: A potential ligand for the ion conductance modulator of the acetylcholine receptor. Proc. Natl. Acad. Sci. USA 74: 2172-2176, 1977.

Habermehl, G., Andres, H., Miyahara, K., Witkop, B., and Daly, J. W.: Synthese von Pumiliotoxin C. Ann Chem.: 1577-1583, 1976.

Daly, J. W., Brown, G., Mensah-Dwumah, M., and Myers, C.: Classification of skin alkaloids from neotropical poison-dart frogs (Dendrobatidae). Toxicon, press.

Mensah-Dwumah, M. and Daly, J. W.: Pharmacological activity of alkaloids from poison-dart frogs (Dendrobatidae). Toxicon, in press.

Albuquerque, E. X. and Daly, J. W.: Batrachotoxin, a selective probe for channels modulating sodium conductances in electrogenic membranes. In Cuatrecasas, P. (Ed.): The Specificity and Action of Animal, Bacterial and Plant Toxins. Receptors and Recognition, Series B, Volume 1. London, Chapman and Hall. 1977, pp. 299-338.

Contracts

Contract No. NO1-AM-4-2203: Isolation of Frog Toxins: $6,000; 1 man year.

Extracts of dendrobatid frogs provided by NIH have been fractionated to yield further quantities of batrachotoxins, histrionicotoxins, pumiliotoxins, and gephyrotoxins for pharmacological evaluation by NIH researchers. Structures of further congeneric alkaloids were determined by spectroscopic analysis. Derivatives of other more unique alkaloids have been prepared and attempts to crystallize these derivatives for X-ray analysis have been made.

Contract No. NO1-AM-7-2212: Synthesis of Membrane-active Tropane and Granatane Derivatives: $9,992; 1 man year.

Synthesis of tropane and pseudopettetierine with side chains at the 1, 2, and 3 position has to be initiated.

| SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space) | U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT** | PROJECT NUMBER Z01 AM 19401-12-LC |
|---|---|---|

PERIOD COVERED

July 1, 1976 through September 30, 1977

TITLE OF PROJECT (80 characters or less)

Synthetic Photochemistry

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: Bernhard Witkop Chief, Laboratory of Chemistry LC-NIAMDD

COOPERATING UNITS (if any) O. Yonemitsu, Y. Kanaoka and T. Iwakuma, University of Hokkaido, Sapporo, Japan, Masako Nakagawa, Faculty of Pharmaceutical Science, Chiba University, Japan; I. L. Karle, U.S. Naval Research Lab., Wash. D. C.

LAB/BRANCH

Laboratory of Chemistry

SECTION

Metabolites

INSTITUTE AND LOCATION

NIAMDD-NIH, Bethesda, Maryland 20014

| TOTAL MANYEARS: | PROFESSIONAL: | OTHER: |
|---|---|---|
| .5 | .5 | 0 |

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☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The synthesis of novel heterocycles of metabolic significance and with potential for antiviral and CNS activity has been accomplished by photocyclizations and dimerizations.



PHS-6040
(Rev. 10-76)

Project Description:

Objectives: Reactions for the selective modification of simple amino acids in proteins and of purines and pyrimidines in nucleic acids are needed for the study of the composition and function of their active sites. Although extensive interest to develop such reactions has been shown by many investigators, photochemical methods have been elaborated with a number of homo- and heterocyclic models which make it possible to deliver selectively a distinct amount of energy to a system. The photochemical behavior of free and bound aromatic amino acids and the corresponding pharmacodynamic amines have been investigated. Studies continue with new heterocyclic systems.

Methods Employed: Ultraviolet irradiation, flash photolysis thin-layer chromatography, silica gel and ion exchange column chromatography, paper chromatography and electrophoresis, UV, IR, NMR and mass spectroscopy.

Major Findings: In collaboration with Masako Nakagawa of Chiba University, Japan, and with Isabella Karle, Nav. Res. Lab., the stereochemical configuration of a potential metabolite of tryptophan, isolated from the photooxydation of D,L- and L-tryptophan, and characterized and crystallized as the N(1)-carbomethoxy-derivative, has been established as methyl 1-carbomethoxy-2,3,3aα, 8aα-tetrahydropyrrolo[2,3-b]-indole-2β-carboxylate which on alkaline hydrolysis gave (cyclic) β-hydroxy-ψ-trytophan, a presumable intermediate between tryptophan and kynurenine.

Another study with Prof. Y. Kanaoka, Hokkaido University, Japan, and Dr. Isabella Karle, NRL, showed that it is possible to disrupt certain aromatic systems by the photoaddition of methanol or ethanol. In the case of the irradiation of 2,4-dimethylphthalimide in methanol or ethanol the novel product was 2(N),7β-dimethyl,7α-O-ethyl(methyl)-1,3,3aβ, 6,7,7aβ-hexahydroisoindol-1,3-dione, a versatile starting compound for novel hydroaromatic heterocycles.

Significance to Biomedical Research and the Program of the Institute: New approaches to novel heterocyclic systems of potential CNS activity.

Proposed Course: Extension into homo- and heterocyclic systems relating to pharmacodynamic amines and to potential antiviral cage compounds.

Publications: None

| SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space) | U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT** | PROJECT NUMBER Z01 AM 19402-04 LC |
|---|---|---|

PERIOD COVERED

July 1, 1976 - September 30, 1977

TITLE OF PROJECT (80 characters or less)

Interferon Induction by Synthetic Polynucleotides and the Antiviral Activity of Nucleoside Analogs.

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

| | | | |
|---|---|---|---|
| PI: | Paul F. Torrence | Research Chemist | NIAMDD,LC |
| Other: | B. Witkop | Chief, Lab. of Chemistry | NIAMDD,LC |
| | G-F. Huang | Visiting Fellow | NIAMDD,LC |
| | M. Edwards | Technician | NIAMDD,LC |

COOPERATING UNITS (if any)

Erik De Clercq, Rega Instituut, Leuven, Belgium; Robert M. Friedman,LCP/NIAMDD
Morio Ikehara, Osaka University, Osaka, Japan; Leonard Kohn, LBP/NIAMDD
~~Albert M. Bobst, University of Cincinnati, Cincinnati, Ohio~~

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SECTION

Metabolites

INSTITUTE AND LOCATION

NIAMDD, Bethesda, Md. 20014

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The structural and conformational features that govern interferon induction are investigated by the synthesis and evaluation of new polynucleotides. The biochemical basis of the observed stringent structural requirements for polynucleotide interferon inducers is sought by investigation of the cell membrane binding properties of nucleic acids and their ability to inhibit protein syntheis in a cell free system. The antiviral and antimetabolic properties of newly synthesized nucleoside analogs is also under investigation.



PHS-6040
(Rev. 10-76)

Project Description:

Objectives: To synthesize modified nucleosides, nucleotides and polynucleotides. To characterize such materials by physicochemical means and then investigate the relationship of molecular structure and conformation to certain biochemical and biological activities of nucleic acids. The chief goal of this project is to provide 1) nucleic acid analogs which may be useful in understanding the biochemistry of cytolytic and oncogenic viruses and/or 2) the prophylaxis or therapy of viral diseases.

Methods Employed: Thin-layer, paper, ion-exchange, gel and other types of column chromatography, liquid scintillation counting, ultracentrifugation, ultraviolet, visible, infrared and nuclear magnetic resonance, spectroscopy, mass spectroscopy, electron spin resonance, circular dichroism, tissue culture.

Major Findings:

1. Inderferon-inducing ability of a 2'-azido modified polynucleotide. Until now, every modification to the 2'-position of a double-helical RNA has resulted in a total less of interferon-inducing ability. Modifications that have been executed include 2'-H (DNA), 2'-$OCH_3$, 2'-Cl, 2'-F, 2'-O-acetate, DNA-RNA hybrids, and azido in the pyrimidine strand of e. g., poly(A)·poly(U) or poly(I)·poly(C). In collaboration with M. Ikehara and E. De Clercq, we have found that the modified ds-RNA, poly(dIz).poly(C), with an 2'-azido group in place of the 2' hydroxyl of the poly(I) strand, is a potent interferon inducer in both primary rabbit kidney cells and human skin fibroblasts.

2. Complexes of poly(2-azainosinic acid) have been found to be inactive as interferon inducers. Their inactivity appears to be related to the lowered thermal stability of the derived double-helices.

3. As mentioned in the previous report, nitronium tetrafluoroborate/sulfolane provides an access to the nitropyrimidine nucleotides and nucleosides. 5-Nitrouridine 5'-diphosphate, prepared by this approach, is not a substrate for polynucleotide phosphorylase, probably becauses the N-·-H pKa is ∿6, three units below the pH optimum (9) for the M. luteus enzyme. 5-Nitro-2'-deoxyuridine has also been prepared by this approach. We find it to be a potent inhibitor of vaccinia virus replication in both primary rabbit kidney cells and human skin fibroblasts. At 100 µg/ml, this nitronucleoside completely arrested vaccinia virus growth, but even at 1 µg/ml, it effected a 1 $\log_{10}$ reduction in virus yield. 5-Nitro-2'-deoxyuridine and its 5'-monophosphate both inhibited the cytopathic effect of vaccinia virus at 0.1-0.4 µg/ml, but both materials were without effect on herpes simplex-1 induced cylopathogenicity at 100 µg/ml. Both the nitro nucleoside and nucleotide effective blocked the incorporation of [$^{14}$C]-dU into acid insoluble material of PRK cells, but neither compound inhibit [$^{3}$H]-dT incorporation, suggesting that thymidylate synthetase might be a target for these nitro analogs.

4. A wide variety of pyrimidine deoxyribo- and arabinonucleosides were found to markedly inhibit replication of herpes simplex-1 (straid KOS) while another herpes simplex virus (HSV-2, strain 333) was unaffected by the nucleoside analogs. This differential activity appeared closely linked to the presence of viral deoxythymidine kinase in the infected cell, since HSV-1 (KOS) was found to induce dT-dC kinase activity wherease HSV-2 (333) failed to do so.

5. An attempt to elucidate the effects (if any) of nucleic acids on cell membranes has began. First we tried to establish some effect of poly(I)·poly(C) on membrane conformation by determining if it could influence the binding of [$I^{125}$]-thyrotropin to mouse L-cell membranes. Unfortunately, poly(I)·poly(C) complicates the outcome of this assay leading to an apparent binding (filter technique) of polynucleotide to the thyrotropin. The basis for this apparent binding has not been established. We are now attempting to establish all the parameters involved in poly(I)·poly(C) binding to L cell membranes to determine the optimal conditions that could be used to look at a possible membrane effect using the ganglioside labeling pattern technique.

6. In collaboration with Dr. R. M. Friedman we are investigating the ability of modified nucleic acids to enhibit cell-free protein synthesis. Several interesting observations suggest this study. Mouse L cells are normally relatively resistant to induction of interferon by poly(I)·poly(C), but priminy the cells with interferon renders them much more sensitive to induction. This phenomenon, described by Stewart, is of even more interest in view of the studies of Kerr's group, showing that interferon pretreatment of L cells enhances the inhibition of protein synthesis (directed by EMC RNA) in the resulting cell-free extract. We posed the question. Is the "receptor" responsible for triggering interferon synthesis the same as the "receptor" that leads to the inhibition of protein synthesis. It is already established that the ds-RNA mediated inhibition of protein synthesis is not a direct effect of ds-RNA. Rather the ds-RNA promotes the formation of an inhibitor(s) of translation. Could interaction of the ds RNA with a single receptor trigger a cascade of cellular events? If this were true, then we would also have a handle to use to isolate the receptor for interferon induction. Thus far, we have basically confirmed the previous reports in the literature. Aged mouse Ly cells give, after preincubation and dialysis of S-10 extracts, a cell free system that is stimulated 10-40X by EMC RNA. When the cells are treated overnight with interferon (60 units/ml), the resulting extracts become sensitized (10-1000X) to ds-RNA inhibition of protein synthesis (inhibitory $dose_{50}$= $10^{-7}$M). In some ways, inhibition of protein synthesis by nucleic acid analogs very much parallels ability to induce interferon, e. g., DNA's, 2-O-$CH_3$, 2'-F, 2'-Cl analogs all fail to inhibit protein synthesis. In other ways, the parallelism seems to stop. Poly(I)·poly($br^5$C), a potent inducer of interferon, does not appear to lead to inhibition of protein synthesis in this system. This study has thus far looked at only a small number of the nucleic acid analogs that must be evaluated. When the list is complete, we should have a better appreciation of the structural factors controlling both interferon induction and inhibition of protein synthesis by ds-RNA's.

Significance to Biomedical Research and the Program of the Institute: Studies described herein and those in progress are of interest for the following reasons.

1) Modified nucleosides can aid in understanding the biochemistry of viruses; in addition, they are of immediate interest as agents active against viral, and neoplastic disease and, in some instances, as local agents in rheumatoid arthritis.

2) Modified polynucleotides aid in the understanding of protein-nucleic acid interactions and nucleic acid-nucleic acid interactions. These considerations are fundamental for a complete knowledge of cellular biochemistry including on the one hand the regulation of expression of genetic information in eukaryotic cells and, at the more practical level, the induction of interferon and the design of a clinically useful interferon inducer.

Publications:

Torrence, P. F. and Witkop, B. "Design of Polynucleotides that Act As Inducers of Interferon." Protein, Nucleic Acid And Enzyme, 21, 536-543.

De Clercq, E., Torrence, P. F., Fukui, T. and Ikehara, M., "Role of Purine N-3 in the Biologic Activities of Poly(A) and Poly(I)", Nucleic Acids Res. 3, 1591-1601 (1976).

Bobst, A. M., Torrence, P. F., Kouidou, S. and Witkop, B., "The Dependence of Interferon Induction on Nucleic Acid Conformation." Proc. Nat. Acad. Sci. USA, 73, 3788-3792.

De Clercq, E., Edy, V., Torrence, P.F., Waters, J. A. and Witkop, B., "In vitro and In vivo Antiviral Activity of Poly(7-Deazainosinic acid) Derived Complexes." Mol. Pharmacol. 12, 1045-1051 (1976).

Torrence, P. F., De Clercq, E. and Witkop, B., "The Interaction of Poly-xanthylic Acid with Polyadenylic Acid", Biochem. Biophys. Acta 475, 1-6 (1977).

Torrence, P. F., De Clercq, E. and Witkop, B. "Polyadenylic·Polyxanthylic·Polyuridylic Acid Triple Helix". Biochemistry 16, 1039-1043 (1977).

Torrence, P. F., Bhooshan, B., Descamps, J. and De Clercq, E., "Improved Synthesis and In Vitro Antiviral Activities of 5-Cyanouridine and 5-Cyano-2'-deoxyuridine". J. Medicin. Chem, in press.

Torrence, P. F. and De Clercq, E., "Induction and Inducers of Interferons". Pharmacology and Therapeutics, in press.

De Clercq, E., Krajewska, E., Descamps, J. and Torrence, P. F. "Anti-Herpes Activity of Deoxythymidine Analogs: Specific Dependence on Virus-Induced Deoxythymidine Kinase". Mol. Pharmacol, in press.

Torrence, P. F. and Witkop, B. "The Synthesis of Poly(2'-azido-2'-deoxyuridylic acid)" New or Improved Syntheses, Methods or Techniques in Nucleic Acid Chemistry," ed. L. B. Townsend, Academic Press, in press.

Torrence, P. F., Waters, J. A. and Witkop, B. "5-Thiocyanato-2'-Deoxyuridine", New or Improved Syntheses, Methods or Techniques in Nucleic Acid Chemistry, ed. L. B. Townsend, Academic Press, in press.

| SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space) | U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT** | PROJECT NUMBER Z01 AM 19403-04 LC |
|---|---|---|

PERIOD COVERED
July 1, 1976 through September 30, 1977

TITLE OF PROJECT (80 characters or less)

Synthesis and Elucidation of Natural Products

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: Thomas F. Spande Research Chemist LC NIAMDD

COOPERATING UNITS (if any) H.C.J. Ottenheijm, Catholic University, Nijmegen, Holland; T. Tokuyama, Osaka City University, Osaka, Japan; A. N. Radhakrishnan, Christian Medical Hospital, Wellcome Research Unit, Tamil Nadu, India

LAB/BRANCH
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SECTION
Metabolites

INSTITUTE AND LOCATION
NIAMDD-NIH, Bethesda, Maryland 20014

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

This project has been discontinued.



PHS-6040
(Rev. 10-76)

| SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space) | U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT | PROJECT NUMBER ZO1 AM 19408-04 LC |
|---|---|---|

PERIOD COVERED

July 1, 1976 through September 30, 1977

TITLE OF PROJECT (80 characters or less)

Central Nervous System Agents Based on the Piperidine Ring System.

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

| | | | |
|---|---|---|---|
| PI: | J. A. Waters | Research Chemist | LC-NIAMDD |
| OTHER: | E. L. May | Adjunct Professor | Medical College of VA. |
| | A. E. Jacobson | Research Chemist | LC-NIAMDD |
| | W. Klee | Research Chemist | LGCB-NIMH |

COOPERATING UNITS (if any)

| | | |
|---|---|---|
| G. Polansky | Stay-In-Schooler | LC-NIAMDD |
| E. Rodgers | Stay-In-Schooler | LC-NIAMDD |

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SECTION

Metabolites

INSTITUTE AND LOCATION

NIAMDD, Bethesda, Md. 20014

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Heterocyclic, aromatic and cycloalkyl 4-piperidinol esters and related piperidines have been synthesized for study of their action on the central nervous system. Several compounds displayed analgesic activity in the codeine-morphine range in the mouse hot-plate assay. In structure-activity comparisons in this system, aromatic esters exhibited $+E_s-\Pi$ substituent effects. In general, the piperidinol esters showed no morphine-like physical dependence capacity in monkeys, acting neither as typical narcotic agonists nor antagonists. In rat brain homogenates in vitro, they displayed marginal to no binding to the opiate receptor.



PHS-6040
(Rev. 10-76)

Project Description

Project No. ZO1 AM 19408-04 LC

Objectives: To design and synthesize compounds from the precursor 4-piperidinol and to assay them for central nervous system activity, including analgesic activity, physical dependence liability and stereospecific binding to the opiate receptor.

Methods Employed: Numerous synthetic procedures have been used to modify the various positions of the piperidine ring and to prepare heterocyclic, aromatic and cycloalkyl carboxylates. Pharmacological and biochemical assays were carried out using established procedures.

Major Findings: Several compounds exhibited potency in the codeine-morphine range in the mouse hot-plate assay. Some activity has also been shown in the mouse p-phenylquinone abdominal stretch and tail-flick assays. In general, these esters showed no morphine-like physical dependence capacity in single dose suppression experiments in monkeys. Typical narcotic agonist or antagonist activities were absent. Binding to the opiate receptor in vitro was marginal or absent.

Significance to Biomedical Research and Program of the Institute: Analgesics, which lack the numerous side-effects effects of morphine, especially physical dependence capacity, and narcotic antagonists are needed for clinical use in relief of pain and coping with the drug addiction problem.

Proposed Course: Radioactive substrates and stereoisomers of selected compounds will be prepared for the study of potenial non-opiate type receptor interactions and central nervous system activities.

Publications:

J. A. Waters, "Pyrrole Esters of Tropanols and Related Structures as Analgesics", J. Med. Chem., in press.

J. A. Waters, "Heterocyclic and Piperonylic Acid Esters of 1-Methyl-4-piperidinol As Analgesics", J. Med. Chem., in press.

| SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space) | U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT** | PROJECT NUMBER Z01 AM 19409-02 LC |
|---|---|---|

PERIOD COVERED

July 1, 1976 through September 30, 1977

TITLE OF PROJECT (80 characters or less)

The Synthesis of Nucleosides with Possible Antiviral Properties

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

| | | | |
|---|---|---|---|
| PI: | Thomas F. Spande | Research Chemist | NIAMDD,LC |
| OTHER: | Paul Torrence | Research Chemist | NIAMDD,LC |
| | Michael Edwards | Research Chemist | NIAMDD,LC |

COOPERATING UNITS (if any)

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SECTION

Metabolites

INSTITUTE AND LOCATION

NIAMDD, Bethesda, Md. 20014

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

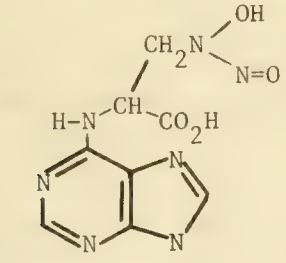
The synthesis of the alanosine derived nucleoside and nucleotide (Ia and Ib) is being pursued in order to determine its effect on adenosine biosynthesis and in order to elucidate the antiviral and antitumor action of alanosine itself.



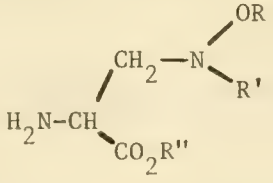
PHS-6040
(Rev. 10-76)

Project Description:

Objectives: The synthesis of 1 is in progress. Structure 1 has been proposed (Gale) as the actual species through which the antiviral and antitumor substance, ãianosine (2) manifests its action in blocking the biosynthesis of adenosine.



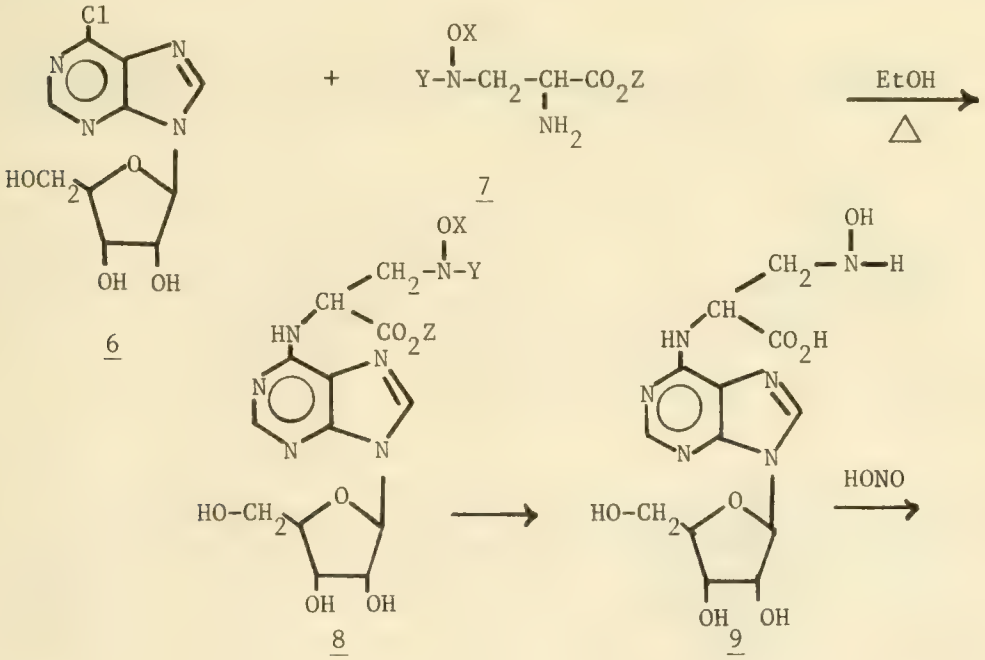
1



2 R=H, R'=NO R"=H
3 R=THP R'=BOC R"=H
4 R=THP R'=BOC R"=Et
5 R=THP R'=BOC R"=$^{t}$Bu

THP = ; BOC = $^{t}$Bu-O-C

The synthesis proposed features as its key step, the reaction between the chloropurine 6 below and appropriately blocked β-hydroxylamino α-amino acids (7)



6

7

8

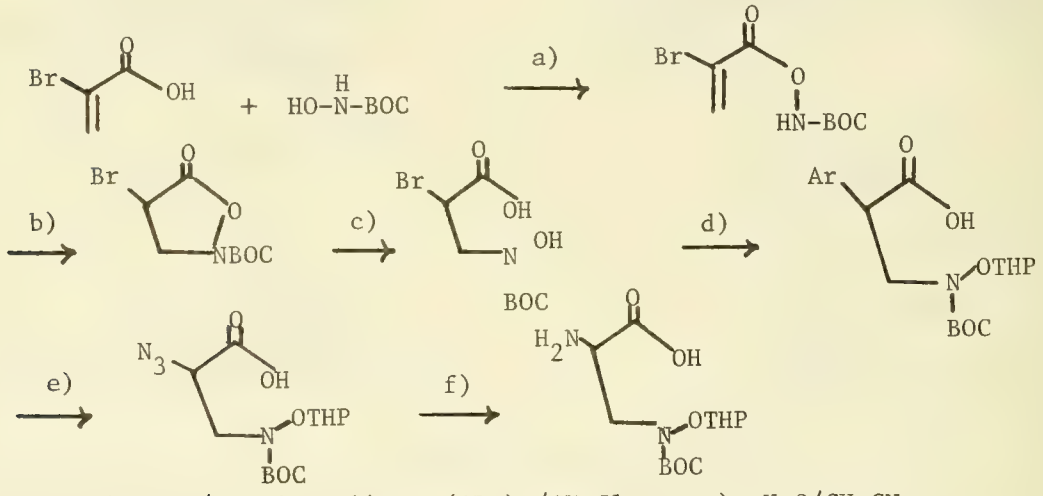
9

Subsequent steps of deblocking and nitrosation should produce 1.

Methods Employed: Ir and PMR spectroscopy, mass spectrometry, thin-layer and column chromatography.

Major Findings: Practical syntheses of the required blocked alanosine precursors 3-5 have been accomplished by the routes below:

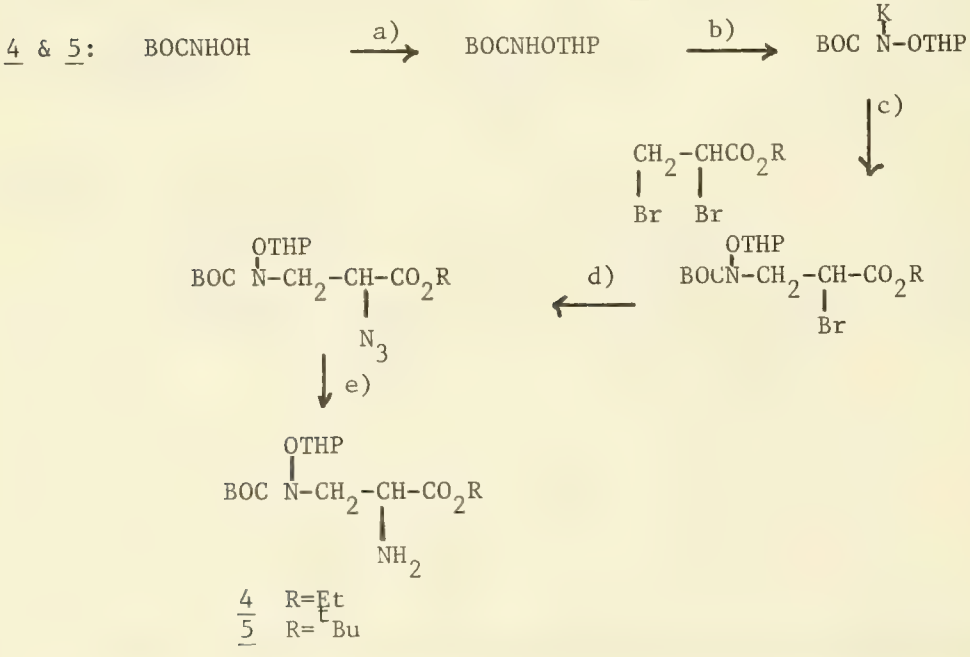
Scheme 1:



a) $DCC/CH_2Cl_2$ b) $EtN(iPr)_2/CH_2Cl_2$ c) $H_2O/CH_3CN$

d) /TFA/$CH_2Cl_2$ e) $LiN_3$/DMF f) Zn/HOAc

Scheme 2



4 R=Et
5 R=$^t$Bu

a) , $ZnCl_2$, 60° b) $KOBu^t/{}^tBuOH$ c) 10% xs $KOBu^t$ in $^tBuOH$,

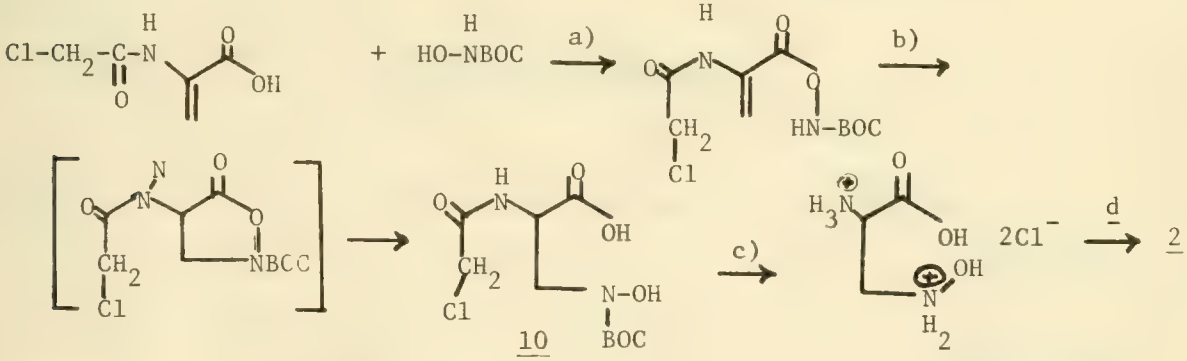
d) $LiN_3$/DMF, 60° e) Zn/HOAc for 4
Zn/HOAc/hexane for 5

1) The reaction of 6 and the blocked amino acid derivative 3 fails even with lengthy reflux periods. This is surprising in view of previous reports on the reaction of amino acids with 6.

2) The reaction of the blocked amino ester 4 with 6 proceeds slowly but gives a satisfactory yield of the intermediate 8 (X=THP, Y=BOC Z=Et) which was completely characterized (IR, UV, NMR, M.S.). The BOC and THP groups could be removed with HCl/ØH; however, side reactions intervened on attempted saponification of the ethyl ester. For this reason we chose to use the blocked derivative 5 where all 3 blocking groups should be removable with HCl/ØH.

3) The reaction of 6 with the tertiary butyl ester 5 proceeded at approximately the same rate as 4. We are currently studying the deblocking of the resulting adduct.

An alanosine synthesis by the route below is being worked on (with M. Edwards)



a) $DCC/CH_2Cl_2$ b) $EtN(iPr)_2/CH_3CN$ c) 6N HCl, 100°

d) $NaNO_2$/HOAc

An improved synthesis of α-chloroacetamidoacrylic acid has been devised. The cyclization, while less facile than the α-bromoacrylic acid derivative, can be made to go in 50-60% yield to give, after isolation, (10) the product of spontaneous hydrolysis. We are currently studying the hydrolysis of this product.

Significance to Biomedical Research and the Program of the Institute:

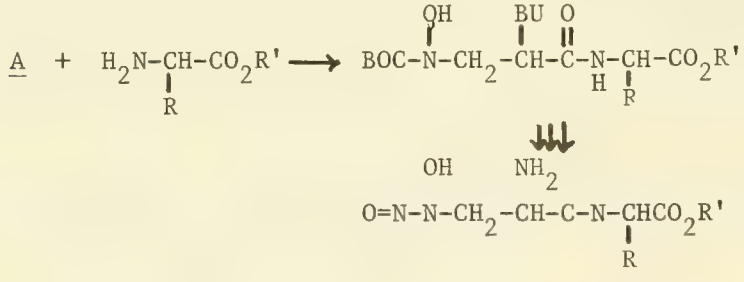
1) There should be much interest in having available sizeable quantities of 1 for testing as an antiviral and antitumor agent.

2. Gales' hypothesis on the mode of action of alanosine could be confirmed if synthetic material were available for study.

3) The action of alanosine could be made more specific-(alanosine affects mainly adenosine monophosphate biosynthesis but also uridine monophosphate biosynthesis to a lesser extent by inhibition of aspartate transcarbamylase.

Proposed Course: If 1 can be made by this route and proves to be interesting pharmacologically, we will attempt a synthesis of 1 from the optically active L amino ester derivative. Either resolution of 5 or an asymmetric synthesis of an appropriate derivative could be used.

The synthetic utility of the reactive isoxazolidinone system (Scheme 1, structure A) should be thoroughly investigated. Our cyclization provides a convenient, nearly quantitative synthesis of this system, recently found to recur in a number of legume seeds. A or variations could be used in a synthesis of N-terminal alanosyl peptides as it is a very reactive acylating agent.



Such peptides would be analogs for N-terminal aspartic acid containing peptides.

Publications

Ottenheijm, H. C. J., Herscheid, J. D. M., Kerkhoff, G. P. C. and Spande, T. F. Approaches to Analogues of Dehydrogliotoxin. 6. An Efficient Synthesis of a Gliotoxin Analogue with Anti-Reverse Transcriptase Activity. J. Org. Chem. 41, 3433 (1976).

| SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space) | U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT** | PROJECT NUMBER Z01 AM 19600-12 LC |
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PERIOD COVERED
July 1, 1976 through September 30, 1977

TITLE OF PROJECT (80 characters or less)

Oxidation Mechanisms in Metabolic Processes

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: Louis A. Cohen Chief, Sec. on Biochemical Mechanisms LC, NIAMDD

COOPERATING UNITS (if any)

None

LAB/BRANCH
Laboratory of Chemistry

SECTION
Biochemical Mechanisms

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20014

| TOTAL MANYEARS: | PROFESSIONAL: | OTHER: |
|---|---|---|
| 0 | 0 | 0 |

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

This project has been temporarily discontinued.

| SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space) | U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT** | PROJECT NUMBER Z01 AM 19601-10 LC |
|---|---|---|

PERIOD COVERED

July 1, 1976 through September 30, 1977

TITLE OF PROJECT (80 characters or less)

Chemical Modification and Cleavage of Proteins

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: Louis A. Cohen Chief, Sec. on Biochemical Mechanisms LC NIAMDD

COOPERATING UNITS (if any)

None

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Laboratory of Chemistry

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Section on Biochemical Mechanisms

INSTITUTE AND LOCATION

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

This project has been temporarily discontinued.



PHS-6040

| SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space) | U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT** | PROJECT NUMBER ZO1 AM 19602-11 LC |
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PERIOD COVERED

July 1, 1976 through September 30, 1977

TITLE OF PROJECT (80 characters or less)

Cleavage of Peptide Bonds by Intramolecular Participation

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: Louis A. Cohen Chief, Sec. on Biochemical Mechanisms LC NIAMDD

COOPERATING UNITS (if any)

None

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

This project has been temporarily discontinued.



PHS-6040
(Rev. 10-76)

| SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space) | U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT** | PROJECT NUMBER ZO1 AM 19603-01 LC |
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PERIOD COVERED

July 1, 1976 through September 30, 1977

TITLE OF PROJECT (80 characters or less)

Fluoro Analogs of Enzyme Substrates

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

| | | | |
|---|---|---|---|
| PI: | Louis A. Cohen | Chief, Sec. on Biochemical Mechanisms | LC NIAMDD |
| OTHER: | Kenneth L. Kirk | Research Chemist | LC NIAMDD |
| | John C. Reepmeyer | Senior Staff Fellow | LC NIAMDD |

COOPERATING UNITS (if any)

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Erik DeClercq, Leuven, Belgium
D. C. Klein, NICHD
B. Highman, Jefferson, Arkansas

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Ring-fluorinated imidazoles show widespread and significant activity as enzyme substrates. 2-Fluorohistidine, but not the 4-fluoro isomer, is repidly incorporated into newly synthesized animal or bacterial protein, in place of histidine. The resulting enzymes may be structurally intact, but some cannot function catalytically because the pK of the imidazole ring has been greatly depressed. 2-Fluorohistidine also serves to produce leucopenia, temporary regression of the mouse spleen, thymus, lymph nodes, and hypocellularity of the bone marrow. In mice infected with leukemia virus P-388, this analog prevents the normally massive increase in leukocyte levels and increases survival time up to 50%. Ultimate death may be due to causes other than leukemia. This anti-leukemic action may be unrelated to production of false protein, and may result from a block in the utilization of histidine as a one-carbon donor in purine biosynthesis. The block would result from the powerful inhibition of urocanase by 2-fluorourocanic acid, a metabolite of 2-fluorohistidine.

Project No. ZO1 AM 19603-01 LC

Project Description:

Investigation of the biochemical and pharmacological properties of ring-fluorinated imidazoles, as tools for the study of enzyme structure and function, and as potential therapeutic agents.

Objectives: Fluoro analogs of steroids, purines, pyrimidines and amino acids have been synthesized and tested, several having been found valuable in arthritis and cancer chemotherapy. A larger number have not been prepared because of the limited routes available for the introduction of fluorine. Despite the ubiquitious role of imidazoles in biological systems (RNA, DNA, histidine, histamine, etc.), no fluorinated imidazole had yet been prepared. The initial purpose of this work was to develop a general method suitable for the preparation of fluoroimidazoles, to prepare a series of such compounds, and to test their abilities to serve as enzyme substrates or inhibitors and as replacement for histidine in proteins or for purines in DNA and RNA. To evaluate fluoroimidazoles as antileukemic, antiviral, and immunosuppressive agents.

Methods Employed: Various enzyme assays, enzyme fractionation and purification methods are employed. Automatic cell counters to determine leukocyte levels. Standard techniques for manipulation of laboratory mice. Culture of yeasts and E. Coli.

Major Findings: Administration of a nonlethal dose of 2-fluorohistidine (2-FHis) to mice produces a time- and dose-dependent leucopenia, temporary regression of the spleen, thymus, and lymph nodes, and hypocellularity of bone marrow. Administration of the histidine analog (150-250 mg/kilo) to mice infected with P-388 leukemia virus prevents the normally massive increase in leukocyte levels and increases survival time up to 50%. Ultimate death of the mice may be due to causes other than the orginal leukemia, a phenomenon now under investigation. We had shown previously that 2-FHis is rapidly incorporated into new protein (in place of histidine) in all murine organs, and it was assumed initially that the effect on leukocyte levels and on leukemia was a result of the biosynthesis of false protein. However, simultaneous administration of histidine or inhibitors of protein synthesis failed to counteract the effects of 2-FHis, these results suggesting that other inhibitory pathways may be involved. In an earlier study [C. B. Klee, L. E. La John, K. L. Kirk, and L. A. Cohen, BBRC, 75, 674 (1977)], 2-fluorourocanic acid, the first product of the catabolism of 2-FHis, was found to be a potent inhibitor of urocanase, the second enzyme in the pathway for histidine degradation. Since it is known that C-2 of the imidazole ring of histidine can be ultimately incorporated into purine bases via N-formyltetrahydrofolate, 2-fluorourocanic acid may serve to retard the utilization of histidine as a one-carbon source for purine biosynthesis; 2-FHis would then function as an in vivo source of 2-fluorourocanic acid. Most anti-leukemic agents in current use also retard purine biosynthesis by inhibition of dihydrofolate reductase and fluorourocanic acid may be retarding an earlier step in the same biosynthetic sequence. This hypothesis is strengthened by the observation that

2-FHis retards the overall growth of E. coli, but does not block the division of preexisting multinuclear cells. Whether histidine serves as an obligatory or secondary one-carbon donor has never been established; 2-FHis and 2-fluorourocanic acid, the first useful inhibitors of histidine catabolism in mammalian systems, may help settle the question.

Studies are continuing on the incorporation of 2-FHis into bacterial and animal protein, with the particular goal of isolating active or inactive enzymes containing fluorine; efforts to obtain biosynthetic fluorohemoglobin are also in progress.

Significance to Bio-medical Research and the Program of the Institute: Studies to date indicate fluoroimidazoles to have a variety of properties in biological systems: enzyme inhibitors, histamine agonists, analogs of histidine, antibacterial, antiviral, and antileukemic agents. These compounds have already proved their usefulness in studies of enzyme structure and function, as inhibitors of histidine metabolism, inhibitors of purine biosynthesis, involvement in cell division, etc. The possibility of therapeutic use for one of more of these compounds should also be considered.

Proposed Course: Additional fluoro analogs are being synthesized for biological evaluation. More detailed studies with enzymes are in progress, as well as efforts to isolate fluorine-containing proteins. The mechanism of the antileukemic effect is being investigated in detail.

Publications:

Kirk, K. L. and Cohen, L. A.: Biochemistry and Pharmacology of Ring-fluorinated Imidazoles. ACS Symposium Series: Biochemistry of the Carbon-Fluorine Bond, 23-36 (1976).

Klein, D. C. and Kirk, K. L.: 2-Fluoro-L-histidine: A Histidine Analog Which Inhibits Enzyme Induction. ACS Symposium Series: Biochemistry of the Carbon-Fluorine Bond, 37-56 (1976).

Klein, D. C., Kirk, K. L., Welles, J. L., Oka, T., Parfitt, A., and Owens, I.S.: 2-Fluorohistidine: An Inhibitor of Enzyme Induction. Mol. Pharm. 12, 720-730 (1976).

Klee, C. B., La John, L. E., Kirk, K. L., and Cohen, L. A.: 2-Fluorourocanic Acid, a Potent Reversible Inhibitor of Urocanase. Biochem. Biophys. Res. Commun., 75, 674-681 (1977).

Creveling, C. R., Kirk, K. L., and Highman, B.: Effect of 2-Fluorohistidine upon Leukocytopoesis in Mice. Res. Commun. in Chem. Path Pharm, 16 507-522 (1977).

Klein, D. C., Weller, J. L., Kirk, K. L., and Hartley, R. W.: Incorporation of 2-Fluoro-L-histidine into Cellular Protein. Mol. Pharm., in press.

Creveling, C. R., Cohen, L. A., Highman, B., and Kirk, K. L.: Inhibition of Leukocytopoesis in P-388 Murine Leukemia. Federation Proc., 36, 975 (1977).

| SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space) | U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT | PROJECT NUMBER ZO1 AM 19604-07 LC |
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PERIOD COVERED

July 1, 1976 through September 30, 1977

TITLE OF PROJECT (80 characters or less)

General Principles of Enzyme Catalysis and Simulation

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

| | | | |
|---|---|---|---|
| PI: | Louis A. Cohen | Chief, Sec. on Biochemical Mechanisms | LC NIAMDD |
| OTHER: | Paul S. Hillery | Senior Staff Fellow | LC NIAMDD |
| | Max Blum | Visiting Fellow | LC NIAMDD |
| | Michael King | Guest Worker | GWU |
| | John C. Reepmeyer | Senior Staff Fellow | LC NIAMDD |

COOPERATING UNITS (if any)

None

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

We have advanced the theory that a major portion of enzymatic catalysis is achieved by activation of the substrate during binding. To support the theory, we have synthesized a large variety of test-tube models which simulate the bound substrate by being frozen in a single, favorable conformation and by having the interacting functions brought into the closest possible juxtaposition. (stereopopulation control). These compounds undergo intramolecular reactions at rates comparable to those catalyzed by enzymes, sometimes even too fast to measure. It has been possible to generate high energy bonds within these models without energy input, simply using the free energy of formation of water and conformational restriction as the driving forces. In current work, models for sulfhydryl enzymes, oxidative phosphorylation and ion pumps are being developed. Techniques are being developed to elucidate molecular conformation in solution, based on $^{13}C$ and $^{19}F$ nmr spectroscopy, as well as computer calculation of conformational energies (CAMSEQ).



PHS-6040

Project No. Z01 AM 19604-07 LC

Project Description:

A study of the principles used by enzymes to accelerate chemical reactions. Model compounds are designed and synthesized which are capable of undergoing various reactions at rates comparable to those of enzymes, and under the same reaction conditions. Recognition of the principal devices used by enzymes will permit the design of more effective drugs and enzyme inhibitors or substrates.

Objectives: The rates of enzyme-catalyzed reactions exceed those of simple test-tube analogs by factors of $10^{10}$-$10^{20}$. In order to account for such a phenomenal effect, model compounds which duplicate one or more of an enzyme's special powers are studied to learn more about the vastly complex protein catalyst. In the belief that the principal enzymatic device is stereo-population control (near perfect orientation of functional groups), model compounds have been designed and synthesized in which rotation of covalent bonds has been severely restricted. By bringing two functional groups into very close proximity and orientation, rates of reaction comparable to those of enzymes can be achieved in the test tube.

Methods Employed: Spectroscopic methods are used to elucidate the structures of synthetic products and to follow rates of reaction. Various chromatographic procedures are employed for analysis and purification.

Major Findings: A series of 6-membered thiolactones has been synthesized in order to compare the kinetics of ring closure to oxygen and the higher energy sulfur lactones. Despite the chemical similarity between the two hetero atoms, serious obstacles were encountered in the synthesis and kinetic analysis in the sulfur series: rearrangement of aromatic ring substituents, air oxidation, and an extremely powerful chelating ability for trace metals. These obstacles have been largely surmounted, and kinetic studies are in progress. The same substrates will be used to study sulfhydryl cleavage of amides, as models for enzymes such as papain. Kinetic studies show that the mechanism for thiolactone formation differs from that for lactone formation, suggesting that serine and cysteine proteases have somewhat different mechanisms for forming their acyl-enzyme intermediates.

Initial exploration has been made in the use of $^{19}F$ nmr as a probe of solution conformation. The temperature dependence observed for the collapse of fluorine doublets indicates rotational barriers considerable higher than predicted for the trialkyl lock. Computer calculations (CAMSEQ) indicate rotational barriers greater than 20 kcal/mole.

Studies with a phenolic groups in the lock position show that the highly hindered side chain is still capable of free rotation, but that the solvated phenolate ion is bulky enough to provide significant rate enhancement. Such models, in which the bulk of the regulating group can be controlled by pH will be used to simulate the ion pump hypothesis for oxidative phosphorylation.

The synthesis of models for acylimidazole formation has been achieved, although synthesis has been complicated by rearrangements involving participation of the imidazole ring. Kinetic studies on the hydrolytic ring opening of cyclic acylimidazoles revealed the rare phenomenon of buffer catalysis of both steps in the hydrolytic process, with buffer concentration determining the rate-limiting step.

Significance to Bio-medical Research and the Program of the Institute: Despite the vast amount of research invested in the elucidation of the pathways of specific enzyme-catalyzed reactions, justification of the rates of these reactions, in comparison with their nonenzymatic counterparts, are still in a primitive state. A sound understanding of the means by which enzymes operate so effectively would lead not only to a better appreciation of biochemical processes in general, but also to a more rational approach to drug design.

Proposed Course: Utilizing the principles of stereopopulation control already developed, additional models will be studied to produce even faster reaction rates than those already observed. The principle will also be extended to other types of reactions, such as hydride transfer, addition/elimination and ion pumps. The control principle will also be used to design conformationally restricted analogs of various drugs with a view to enhancement of activity and reduction of side-effects.

Publications: None

| SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space) | U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT** | PROJECT NUMBER ZO1 AM 19605-01 LC |
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PERIOD COVERED

July 1, 1976 through September 30, 1977

TITLE OF PROJECT (80 characters or less)

Chemistry of Substituted Imidazoles

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

| | | | |
|---|---|---|---|
| PI: | Louis A. Cohen | Chief, Sec. on Biochemical Mechanisms | LC NIAMDD |
| OTHER: | Kenneth L. Kirk | Research Chemist | LC NIAMDD |
| | Y. Takeuchi | Visiting Associate (left Feb. 1977) | LC NIAMDD |
| | Sarah Woods | Guest Worker | Roosevelt Univer. |
| | H. Kimoto | Visiting Fellow (arr. May, 1977) | LC NIAMDD |
| | John C. Reepmeyer | Senior Staff Fellow | LC NIAMDD |
| | Peter Cohen | Guest Worker | LC NIAMDD |
| | R. Jerussi | Guest Worker | LC NIAMDD |

COOPERATING UNITS (if any)

None

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Discovery of the widespread and impressive properties of fluoroimidazoles as biological substrates and analogs has provided the impetus for the synthesis of other substituted imidazoles. Methods for their synthesis, however, were largely unknown and our explorations of resonance and inductive effects in imidazoles provided the basis for development of new synthetic methods. Thus, intermediates for the synthesis of 2,4-difluoroimidazoles have become available for the first time. Development of the triphenylmethyl blocking group for imidazole nitrogen provided the first general route to 2-substituted imidazoles (including histamines and histidines). A novel route to 2-trifluoromethyl-imidazoles (including histidine and histamine) has been discovered, based on the reaction of noncyclic precursors with trifluoroacetic anhydride. These analogs will be very useful in evaluating the significance of an acidic ring NH on the biological properties of the natural imidazoles.

Project No. Z01 AM 19605-01 LC

Project Description:

Development of new methods for the synthesis of fluoro and other analogs of biochemically significant compounds and examination of their properties as enzyme inhibitors and as potential drugs.

Objectives: Fluoro analogs of steroids, purines, pyrimidines and amino acids have been synthesized and tested, several having been fund valuable in arthritis and cancer chemotherapy. A larger number have not been prepared because of the limited routes available for the introduction of fluorine. Despite the ubiquitous role of imidazoles in biological systems (RNA, DNA, histidine, histamine, etc.), no fluorinated imidazole had yet been prepared. The initial purpose of this work was to develop a general method suitable for the preparation of fluoroimidazoles, to prepare a series of such compounds, and to test their abilities to serve as enzyme substrates or inhibitors and as replacement for histidine in proteins or for purines in DNA and RNA. Discovery of the widespread and significant biological properties of fluoroimidazoles provided the impetus for the synthesis of other substituted imidazoles.

Methods Employed: Ultraviolet irradiation is used as the key step in the introduction of fluorine. Infrared, ultraviolet, mass, and nmr spectroscopy are used to follow the course of reactions and to elucidate product structure. Various chromatographic procedures are used for analysis and purification of materials.

Major Findings: The unusual physical and chemical properties of substituted imidazoles, which are so intimately related to their biological properties, have been the subject of further investigation. We have demonstrated that the exceptionally low basicities of fluoroimidazoles are not anomalous, since the $pK_1$ values of ten fluoroimidazoles can be included with seventy other mono- and polysubstituted imidazoles in a correlation with aromatic ortho substituent constants [L.A. Cohen and S. Takahashi, JACS, 95, 443 (1973)]. Values of $pK_2$ also correlate well with this scale. Thus, the acidic or basic pK of any new imidazole can now be predicted with high accuracy. The generally accepted correlations with meta sigma constants must now be considered invalid.

The anomalous nmr spectra of haloimidazoles have also been explained. For protons attached to $sp^3$ carbon, chemical shifts are determined by the electronegativities of chain substituents; for protons on aromatic and heteroaromatic carbon, however, only the pi-electron density is significant and values for imidazole protons have been correlated successfully with the resonance components of aromatic $\mathcal{T}$ constants [L. A. Cohen and W. M. Jones, JACS, 85, 3402 (1963)]. Efforts to extend these correlations to $^{13}C$ nmr values are in progress.

Studies on acid-catalyzed hydrogen isotope exchange in fluoroimidazoles led to the prediction that nitration of these compounds should occur under very mild conditions. Indeed, the prediction has been verified and [intermediates for the synthesis of 2,4-difluoroimidazoles have become available for the first time. Development of the triphenylmethyl (trityl) blocking groups for imidazole nitrogen represents a major advance in synthetic imidazole chemistry,

providing the first general route to 2-substituted imidazoles (including histamines and histidines) and to difficultly accessible 4-substituted imidazoles.] The procedure involves generation of a carbanion at C-2 of the N-tritylimidazole with butyl lithium, reaction with an electrophilic reagent, and removal of the blocking group in dilute acetic acid. A novel route to 2-trifluoromethylhistidine has been discovered, based on the Bamberger cleavage of histidine with benzoyl chloride and reaction of the product with trifluoroacetic anhydride. This analog of histidine will be very useful in evaluating the significance of an acidic ring NH on the biological properties of the amino acid. Additional members of the imidazole trimer series have been synthesized, including tristidine (a cyclic trimer of histidine); these compounds are being evaluated as mutagenic agents. Photochemical fluorination, developed orginally to make fluoroimidazoles, has been extended to other ring systems: e. g., 2-flucromorphine has been synthesized from 2-nitromorphine.

Significance to Bio-medical Research and the Program of the Institute
Fluoroimidazoles have already been found to be powerful tools for the investigation of enzyme structure and function, as inhibitors of metabolic pathways and as potential therapeutic agents. We believe that parallel properties will be observed with other analogs of natural imidazoles, possibly with even greater potency. These analogs are designed to answer specific questions regarding the steric and geometric limitations of natural substrates, the importance of $pK_1$ and $pK_2$ of imidazoles to their biological behavior, and the role of ring current effects.

Proposed Course: Continued efforts will be made to develop synthetic routes to difficultly accessible imidazoles. Studies will be continued in an effort to rationalize the anomalous physical and chemical properties of the imidazole series. Many of the compounds will be evaluated for their biological properties.

Publications:

Y. Kikugawa and L. A. Cohen: Synthesis of N-alkylimidazoles from N-alkyloxazolium salts. Chem Pharm. Bull., 24, 3205-3207 (1976).

Takeuchi, Y., Cohen, P. A., Kirk, K. L., and Cohen, L. A.: Mechanisms for Hydrogen-Deuterium Exchange in Ring-fluorinated Imidazoles. Abstracts, 1977 Annual Meeting, American Chemical Society, in press.

| SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space) | U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT | PROJECT NUMBER ZO1 AM 19606-01 LC |
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PERIOD COVERED
July 1, 1976 through September 30, 1977

TITLE OF PROJECT (80 characters or less)

Halogenated Biogenic Amines in Biochemistry and Pharmacology

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: Kenneth L. Kirk Research Chemist LC NIAMDD

COOPERATING UNITS (if any)

C. R. Creveling, LC, NIAMDD; L. I. Goldberg, U. Chicago; G. Firnau, McMaster Univer.; D. Murphy, J. Costa, N. Bartek, NIMH; D. C. Joy, D. M. Maher, Bell Labs S. W. Hui, Roswell Park

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Fluorinated analogs of tyramine, dopamine, melatonin, and serotonin (5-hydroxytryptamine) have been synthesized by novel chemical methods, including photo-chemical transformation. Studies with these analogs have provided significant information on the importance of substrate lipophilicity to MAO-A and MAO-B, the role of zwitterionic character in the transport of serotonin, the role of phenolic pK in the activities of dopamine, and the sites of storage of serotonin in tissues.

Project Description:

Development of new methods for the synthesis of halogenated analogs of biogenic amines, study of their physical properties, and evaluation of their behavior in biological systems as antagonists or agonists.

Objectives: Halogenated analogs have proved to be powerful tools for the study of transport, storage, release, and action of biogenic amines, since they simulate the geometry of the natural compounds so well. Continued studies should provide significant information about the roles of these essential metabolic species and possibly lead to therapeutic use.

Methods Employed: Ultraviolet irradiation and various chemical methods are used for the introduction of halogen into biogenic amines. Infrared, ultraviolet, mass, and nmr spectroscopy are used to follow the course of reactions and to elucidate product structure and purity. Various chromatographic procedures are used for purification and analysis of materials. Electron energy-loss spectroscopy is used to detect and localize fluorine-containing materials in tissues.

Major Findings: 5-Hydroxytryptamine (5-HT) is a substrate for monoamine oxidase A (MAO-A), but not for MAO-B. In contrast, 6-fluoro-5-HT and 4,6-difluoro-5-HT are substrates for both the A and B enzymes. Tyramine is a substrate for both MAO-A and MAO-B, but becomes a substrate only for the B enzyme following ring fluorination. It has been proposed that MAO-B requires more lipophilic substrates than does MAO-A, and the results with the more lipophilic ring-fluorinated analogs appear to support this view.

In studies designed to investigate the structural features necessary for the transport and storage of 5-HT, it has been found that 4,6-difluoro-5-HT is taken up by human platelets at a much higher rate than 5-HT itself. Since the fluorine substituents increase the acidity of the phenolic group without perturbing the basicity of the primary amine, these results appear to support the hypothesis that the zwitterionic form of 5-HT is the actual substrate being transported.

Ring-fluorinated analogs of dopamine (2-, 5-, or 6-fluoro) have been evaluated for dopamine activity *in vivo* (dogs); despite their increased phenolic acidity, all these analogs show activity essentially identical to that of dopamine, suggesting phenol acidity to be a noncritical factor in dopamine activity. The similarity in activities may be more apparent than real, however, since decreased activity could be counterbalanced by more rapid uptake (see above). Appropriate experiments with isolated receptors are in progress.

Electron energy-loss spectroscopy, an efficient method for the detection of low levels of elements below calcium in the periodic table, has been applied for the first time to detect and localize fluoro analogs in biological materials. We had shown previously that tritiated 4,6-difluoroserotonin is taken up efficiently by human platelets with concomitant displacement of endogenous serotonin.

By use of the new spectroscopic technique on air-dried platelet whole mounts, the fluoro analog was localized in the platelet storage vesicles, providing direct evidence that serotonin had been stored in and released from the same vesicles. The combination of electron energy-loss spectroscopy and fluorine as a tracer offers several advantages for substrate localization in tissues: a high signal-to-noise ratio, the virtual absence of random fluorine in living cells, and the frequent simulation of natural substrates by their fluorinated analogs.

Significance to Bio-medical Research and the Program of the Institute: Biogenic amines play a key role in neurotransmission, metabolism, and in control of various physiological processes. Relatively little is known regarding mechanisms of transport, storage, release, and action of these agents. The availability of effective analogs provides a unique set of tools for the study of these mechanisms.

Proposed Course: Additional analogs will be synthesized to help answer specific questions regarding the biochemistry and pharmacology of biogenic amines. Evaluation of the analogs will be continued with a view to development of possible therapeutic agents.

Publications:

Kirk, K. L.: Photochemistry of Diazonium Salts. IV. Synthesis of Ring-fluorinated Tyramines and Dopamines. J. Org. Chem., 41, 2373-2376 (1977).

Kirk, K. L.: Synthesis of Ring-fluorinated Serotonins and Melatonins. J. Heterocyclic Chem., 13, 1253-1256 (1977).

Costa, J. L., Joy, D. C., Maher, D. M., Kirk, K. L., and Hui, S. W.: Subcellular Localization of Fluorinated Serotonin in Human Platelets by Electron Energy-loss Spectroscopy. Proceedings of the Thirty-Fifth Annual EMSA Meeting, in press.

| SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space) | U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT** | PROJECT NUMBER Z01 AM 19801-32 LC |
|---|---|---|

PERIOD COVERED
July 1, 1976 to September 30, 1977

TITLE OF PROJECT (80 characters or less)

Service Functions and Instrumentation

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

| | | | |
|---|---|---|---|
| PI: | D. F. Johnson | Section Chief | LC-NIAMDD |
| Other: | H. Y. C. Yeh | Senior Staff Fellow | LC-NIAMDD |
| | W. R. Landis | Chemist | LC-NIAMDD |
| | P. Parisius | Chemist | LC-NIAMDD |
| | B. Baer | Chemist | LC-NIAMDD |
| | A. Wong | Chemist | LC-NIAMDD |
| | N. Whittaker | Chemist | LC-NIAMDD |

COOPERATING UNITS (if any)

None

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Laboratory of Chemistry

SECTION
Microanalytical Services and Instrumentation

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Md. 20014

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Basic research and service functions are performed by members of the Section. A major mission of the organization involves the instrumental and chemical analyses provided to scientists of the Laboratory of Chemistry, NIH, and to a limited extent to personnel of other government agencies. Approximately 30 of the more common elements and five functional groups are determined on a quantitative basis, using ultramicro, micro, and semi-micro techniques as required. The materials analyzed include organic and inorganic research samples, commercial preparations and various biological specimens. Molecular weights are determined by vapor pressure osmometry in both aqueous and nonaqueous solvents when requested. Instrumental analyses include: GC/MS spectrometry, gas-liquid chromatography, GC with radioactive monitoring, infrared, nuclear magnetic resonance, atomic absorption spectrophotometry, ultraviolet, and flame photometry. Chemical analyses are done by most of the commonly used techniques including: gravimetric, colormetric, gasometric, coulometric, and volumetric. Assistance in the interpretation of spectra is rendered on request.

Project Description: Basic research and service functions are performed by members of the Section. A major mission of the organization involves the instrumental and chemical analyses provided to scientists of the Laboratory of Chemistry, NIH, and to a limited extent to personnel of other government agencies. Approximately 30 of the more common elements and five functional groups are determined on a quantitative basis, using ultramicro, micro, and semi-micro techniques as required. The materials analyzed include organic and inorganic research samples, commercial preparations and various biological specimens. Molecular weights are determined by vapor pressure osmometry in both aqueous and nonaqueous solvents when requested. Instrumental analyses include: GC/MS spectrometry, gas-ligand chromatography, GC with radioactive monitoring, infrared, nuclear magnetic resonance, atomic absorption spectrometry, ultraviolet, and flame photometry. Chemical analyses are done by most of the commonly used techniques including: gravimetric, colormetric, gasometric, coulometric, and volumetric. Assistance in the interpretation of spectra is rendered on request.

During the past year the total number of analyses performed in the microanalytical laboratory was 10,657 roughly a 3% decrease in the total from the last reporting period. This represents a relatively stable level of usage compared to the 19% decrease reflected in the last reporting period. Review of the special types of analyses are shown below;

Analyses Run

| | 1975-76 | 1976-77 | % Change |
|---|---|---|---|
| Carbon | 2857 | 2379 | -16.7 |
| Hydrogen | 2857 | 2379 | -16.7 |
| Nitrogen | 4105 | 4780 | +16.4 |
| Phosphorous | 191 | 320 | +67.5 |
| Sulfur | 95 | 81 | -14.7 |
| Halogens | 375 | 357 | - 4.8 |
| Metals | 133 | 62 | -53.4 |
| Weight Loss | 64 | 31 | -51.6 |
| Rotations | 63 | 20 | -68.3 |
| Miscellaneous | 174 | 248 | +42.5 |
| | 10,914 | 10,657 | - 2.4 |

The distribution of service rendered is LC (20%); NIH, not including LC (66%); and outside agencies (8%). This distribution is comparable to the previous reporting period.

Very little service work was performed on the Perkin-Elmer 421 infrared spectrometer. The bulk of this type of analysis is done by individual investigators on low resolution infracords, and our instrument is only used for special studies. It is retained however because it is the only such instrument available to LC.

The NMR service includes both the Varian A60 and HA-100. The major share of service work is done on the former. Over 200 spectra were run on the A60 by Mr. Benjamin Miller under the guidance of Mr. Noel Whittaker. Additional spectra were run by individual investigators. Mr. Miller also maintains the documentation and stocking of organic chemical stores for the laboratory. As an additional service function in NMR, Dr. Herman Yeh has run 300 spectra on the Varian HA-100 and 30 on a Varian HR-200 (located in a different section). These specialized spectra were, in addition to Dr. Yeh's research program, reported elsewhere.

The nmr service was significantly upgraded during the past year with the acquisition of a JEOL FX-100 pulse Fourier transform spectrometer. This instrument is capable of measuring $^{1}H$, $^{19}F$, and $^{13}C$ nuclei. It will also allow for various spin decoupling modes, auto-T measurement, auto-stocking and spectral simulation.

Over 1000 samples were submitted to Mr. William Landis for mass spectral analysis (Hitachi Perkin-Elmer RMU-6E Mass Spectrometer). Each sample analysis was successfully completed according to the investigator's requests and our instrument's capability. This involved standard qualitative spectra; isotope quantitative mass-ratio measurements; on-scale spectra recorded either/or the visicorder and the pen recorder; and sample mixtures requiring fractional volatility-changing rate separations occuring via the solids probe.

We are continuing to explore the procurement of a compatible computer data-system to be interfaced with the Hitachi P-E mass spectrometer. Presently, two existing systems are being considered, and we are sight-visiting the respective manufacturer's installations in order to determine which data-system is most advantageous to our laboratory needs. Hopefully, in the near future a decision will be made to place a definite order, for the desired data package. This Data System will greatly expand and enhance the existing capabilities of our mass spectrometer for spectral analyses.

The number of samples analyzed are tabulated accordingly:

| | |
|---|---|
| NIAMDD - Laboratory of Chemistry | 1009 |
| N.I.H., other than NIAMDD-LC | 98 |
| Outside the N.I.H. | 41 |
| Total samples analyzed | 1148 |

Mr. Noel Whittaker is the principal operator of the Finnigan GC/MS system. Over 600 solid probe samples were run utilizing both chemical ionization and electron impact. This system is very heavily utilized by investigators of the LC but a limited amount of collaborative work has been carried out with outside groups. Mr. Whittaker is in the process of assembling a low

pressure electron impact source to facilitate running in the CI and EI modes. A second technique under development is the use of field desorption emitters to obtain spectra on nonvolatile samples. Additionally, he does a limited number of IR spectra on the Perkin-Elmer 421 spectrophotometer on an individual request basis.

The Section Chief continues to serve as Project Officer on an NIAMDD contract with the Medical Research Council of England. This contract provides reference steroid compounds to qualified research workers in the United States.

Publications: None

| SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space) | U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT** | PROJECT NUMBER ZO1 AM 19802-04 LC |
|---|---|---|

PERIOD COVERED
July 1, 1976 to September 30, 1977

TITLE OF PROJECT (80 characters or less)

Characterization of the Dehydrogenase-Isomerase Enzymes and Metabolic Pathways in Tetrahymena Pyriformis

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT


| | | | |
|---|---|---|---|
| PI: | Nancy S. Lamontagne | Research Chemist | LC-NIAMDD |
| Other: | David F. Johnson | Section Chief | LC-NIAMDD |


COOPERATING UNITS (if any)
Chester E. Holmlund, Department of Chemistry, University of Maryland, College Park, Maryland

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)


Pregnenolone was identified as the major product when progesterone was incubated with cultures of Tetrahymena pyriformis. This transformation is the first demonstration of reversal of the 5-ene-3β-hydroxy ⇄ 5-ene-3-ketone ⇄ 4-ene-3-ketone pathway in an intact organism. Our objective is to characterize the nature of this dehydrogenase-isomerase system of T. pyformis by purification of the dehydrogenase and isolation of the pathway intermediates. A growth inhibition study will also be done to investigate the hypothesis that these transformations are related to the toxicity of steroids to this protozoan.




PHS-6040
(Rev. 10-76)

Project Description: The protozoan Tetrahymena pyriformis has steroid dehydrogenases that are similar in functional properties to the dehydrogneases of higher eucaryotes. Specifically, our prior identification of 4-androstene-3β,17β-diol and pregnenolone as transformation products from the incubation of T. pyriformis with testosterone and progesterone, respectively, indicated that this protozoan possesses a dehydrogenase-isomerase system that can isomerize a $\Delta^4$-steroid to a $\Delta^5$-steroid and reduce the C-3 carbonyl to a 3β-hydroxyl. The characterization of this system is being pursued in several ways.

The β-hydroxysteroid dehydrogenase (β-HSDH) is obtained as a crude supernatant from a 100,000 x g centrifugation of ruptured cells. Column techniques such as pseudo-affinity chromatography on a blue sepharose matrix and molecular sieving on a Bio-Gel-5A are being used to purify the β-HSDH. Although the amount of this enzyme activity in T. pyriformis is small, the development of a quick spectrophotometric assay and the availability of large fermentors is making the purification feasible. Once a sufficient amount of β-HSDH activity is partially purified it will be used for kinetic and substrate specificity studies.

The incubation of T. pyriformis with $C^{14}$ or $H^3$-labeled progesterone, testosterone, pregnenolone, and 5-androstene-3β,17β-diol is being done in the hope of isolating the intermediate(s) of the β-dehydrogenase $\Delta^4$-,$\Delta^5$-isomerase pathway. Qualitative and quantitative identification of these intermediates by column, thin-layer, and gas chromatography and other analytical techniques could indicate whether this pathway has any reversibility in T. pyriformis and/or whether it operates via the same sequence of steps as have been determined in higher animals.

Cultures of T. pyriformis will be grown in the presence of varied concentrations of unlabeled steroids. Growth inhibition will be measured by cell counting in a hemocytometer. A fixative technique has been developed to immobilize the protozoa and Trypan blue will be used to distinguish between viable and nonviable intact cells.

Publications: Lamontagne, N. S., Will, A. R., Johnson, D. F., and Holmlund, C. E.: The Conversion of Progesterone to Pregnenolone by Tetrahymena pyriformis. J. of Steroid Biochem. 8: 329, 1977.

| SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space) | U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT** | PROJECT NUMBER Z01 AM 19803-04 LC |
|---|---|---|

PERIOD COVERED
July 1, 1976 to September 30, 1977

TITLE OF PROJECT (80 characters or less)

Applications of NMR in Biochemical and Biological Systems

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

| | | | |
|---|---|---|---|
| PI: | H. J. C. Yeh | Research Chemist | LC-NIAMDD |
| **Other:** | **D. F. Johnson** | **Section Chief** | **LC-NIAMDD** |
| | **H. M. Fales** | **Section Chief** | LC-NHLBI |
| | D. M. Jerina | Section Chief | LC-NIAMDD |
| | H. Yagi | Research Chemist | LC-NIAMDD |
| | A. E. Jacobson | Research Chemist | LC-NIAMDD |
| | W. A. Klee | Research Biochemist | LGCB NIMH |
| | M. Sato | Visiting Fellow | LCP-NIAMDD |
| | I. Morishima | Visiting Scientist | LCP-NIAMDD |
| | Scott Chiang | Visting Fellow | LC-NIAMDD |
| | L. Levenbook | Research Biochemist | LPB-NIAMDD |
| | E. A. Sokoloski | Research Chemist | LC-NHLBI |

COOPERATING UNITS (if any)
R. S. Wilson, M. Koreeda, and P. D. Moore (The Johns Hopkins University); M. M. King (The George Washington University); G. O. Dudeck (Harvard University); P. S. Chen (University of Zurich); R. Katz (Mid-Atlantic Research Inst., Bethesda, Md.).

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The purpose of this project is to develop nuclear magnetic resonance methods for elucidating molecular structure and for studying the interactions within and between molecules, especially those of biological interests.

Project Description: Basic information on the structure and molecular interactions of biologically important materials is of significance to biochemical and biological research throughout NIH. One of the most promising approaches in obtaining such information is through the use of nuclear magnetic resonance. The purpose of this project is to develop nuclear magnetic resonance methods for studying molecular structures and molecular interactions in biochemical and biological systems.

Various NMR methods (e.g. selective decoupling experiment, lanthanide shift experiment, etc.) have been used successfully to assign the structure of compounds such as the dihydrodiols (derived from bacterial oxidation of the carcinogens benzo[a]pyrene and benzo[a]anthracene), the halomorphides (narcotic drugs) and a unique galactoside from male drosophile melanogaster.

$[^{31}P]$-$^{1}H$ NMDR technique coupled with pD titration has been used to study the interaction between phosphate containing molecules, 4-deoxypyridoxine phosphate and monomethyl phosphate, and a number of structurally related biogenic amines. The difference in the phosphorus chemical shifts with and without amine in deuterated water indicated that optimal biogenic amine-phosphate interaction appears to require the presence of both a catechol ring and a β-hydroxyl group in the ethylamine side chain. This is in good agreement with in vitro pharmacological measurements of biogenic amine retention by storage vesicles of rat heart adrenergic nerve terminals, indicating that phosphate could possibly be involved in binding of biogenic amines in these vesicles. The in vitro model presented could provide a rapid and efficient method for biological evaluation of various drug-receptor systems involving phosphate-amine interactions.

Porphyrins and metalloporphyrins are important components of several biological macromolecules, and many of the biologically significant properties of these molecules are directly related to the interaction among the central metal and the chelating nitrogen atoms, and among the central metal and the ligands. Such interactions appear to be ideally suited for investigations by various nuclear magnetic resonance spectroscopies.

The binding of cyanide ion, imidazole and the mixed ligand to ferriprotoporphyrin IX·Cl (hemin) in DMSO-$d_6$ has been studied by $^{1}H$ nuclear magnetic resonance. The results show that the cyanide ligation to hemin is a two step process, with first the formation of a previously unknown monocyano adduct of hemin and then followed by a dicyanohemin in completion. Binding of imidazole to hemin, however, is a concerted process which only forms diimidazolehemin product. The relative stabilities of various hemin-ligand complexes were determined as $K_{CN,Im} \geq K_{diCN} > K_{diIm} \sim K_{monoCN} >> K_{monoIm}$. The iron(III) of intermediary species, monocyanohemin, was determined as low spin iron (S = 1/2) while monoimidazolehemin has been reported as high spin iron (S = 5/2). The rate of exchange among different hemin species was slow on the NMR time scale, at a rate slower than 160 $sec^{-1}$ at 65°.

We have used the $[^{15}N]$-$^{1}H$ INDOR experiment to obtain the $^{15}N$ spectra of $^{15}N$-labeled meso-tetraphenylporphyrin at several temperatures. Our results

at -80° show the presence of two kinds of nitrogen in the TPP free base, suggesting that the two imino hydrogen atoms are bound to opposite nitrogen atoms. These nitrogens become equivalent at 30° as a result of intramolecular rapid exchange of the imino protons among the four nitrogen atoms.

Using the [$^{111}$Cd]-$^{1}$H and [$^{15}$N]-$^{1}$H INDOR experiments we are able to obtain the $^{111}$Cd and $^{15}$N spectra of $^{111}$Cd meso-tetraphenylporphyrin-$^{15}$N in $CDCl_3$ at 5-10 mg quantity. The one bond $^{111}$Cd-$^{15}$N coupling constant was first measured being 144 Hz. Currently we are investigating the effect of ligands on the $^{15}$N and $^{111}$Cd NMR spectra.

Nuclear magnetic resonance has been used to obtain information about iron-protein and iron-ligand bindings in hemerythrin, a nonheme iron protein responsible for oxygen transport in certain species of marine invertebrates. The results of our preliminary study indicate a significant difference between oxyhemerythrin and deoxyhemerythrin in the aromatic region of their $^{1}$H NMR spectra, suggesting that the amino acid residues bearing the aromatic side chains, e.g. histidine, tyrosine and tryptophan, are most likely to be involved in binding to the iron atom of which the electronic state changes as the oxygen molecule is removed from oxyhemerythrin. We are currently investigating the effect of ligands on the NMR spectra of hemerythrin at various oxidation states in order to unravel the structure at the active site. Investigation of these systems by $^{13}$C NMR is also planned.

Publications: Yeh, H. J. C., Wilson, R. S., Klee, W. A. and Jacobson, A. E.: α- and β- Halomorphides: stereochemistry, analgesic potency, toxicity and their interaction with narcotic receptors in vitro. J. Pharmaceut. Sci. 65: 902-904, 1976.

King, M. M., Yeh, H. J. C. and Dudeck, G. O.: Nitrogen NMR spectroscopy: application to some substituted pyrroles. Org. Mag. Resonance 8: 208-212, 1976.

Koreeda, M., Moore, P. D., Yagi, H., Yeh, H. J. C. and Jerina, D. M.: Alkylation of polyguanylic acid at the 2-amino group and phosphate by the potent mutagen(±)-7β,8α-dihydroxy-9β,10β-epoxy-7,8,9,10-tetrahydrobenzo[a]pyrene. J. Am. Chem. Soc. 98: 6720-6722, 1976.

Yeh, H. J. C., Sato, M. and Morishima, I.: $^{1}$H and $^{15}$N NMR studies of $^{15}$N-labeled meso-tetraphenylporphyrin. J. Mag. Resonance in press: 1977.

Katz, R., Yeh, H. J. C. and Johnson, D. F.: Biogenic amine and organic phosphate interactions: a proposed in vitro model for study of biological interactions by $^{31}$P nuclear magnetic resonance. Mol. Pharmacol. in press: 1977.

Chen, P. S., Fales, H. M., Levenbook, L., Sokoloski, E. A. and Yeh, H. J. C.: Isolation and Characterization of a unique galactoside from male drosophile melanogaster. Biochemistry in press: 1977.

| SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space) | U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT** | PROJECT NUMBER Z01 AM 19804-04 LC |
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PERIOD COVERED

July 1, 1976 to September 30, 1977

TITLE OF PROJECT (80 characters or less)

The Development of Chemical Methods and Compounds for the Study of Biological and Medical Problems

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: Calvin M. Foltz Research Chemist LC-NIAMDD
Other: None

COOPERATING UNITS (if any)

None

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The primary goal of this work is to contribute to the investigation and solution of basic biological and medical problems. This is to be done by the development of chemical methods and reagents for the study of the primary, secondary, tertiary, and quaternary structures of biomacromolecules, by the study, development, and application of organic and photochemical reactions, and by synthesis of selected chemical compounds. Areas of special interest are photocyclization, photorearrangements, chemistry of selenium compounds, biochemistry of selenium, and chemotherapeutic agents.

Project Description: The objective of this project is to make contributions to the investigation and solution of basic biological and medical problems by the application of chemical and physical methods.

Work is planned on the extension of the photocyclization observed with N-chloroacetyltryptophan [Yonemitsu, Cenutti, and Witkop (1966)] to tryptamine and derivatives and homologs of tryptamine. The tricyclic lactams produced are of chemical and potential biological and medical interest. The reaction was extended to non-heterocyclic systems with photolysis of N-chloroacetyl-2-(-naphthyl)ethylamine [Foltz, J. Org. Chem., 36, 24 (1971)]. Additional work is also in progress on the study of the scope, mechanism, and applications of the rearrangement observed on photolysis of 3-(3'-chloro-2'-oxopropyl)-indolin-2-one [Foltz and Kondo, Tetrahedron Letters, 3163 (1970)]. These studies will yield compounds of potential biological and medical interest and possibly methods for the modification and study of biological molecules and structures.

In 1957, the discovery of the fact that Factor 3, which prevented dietary liver necrosis in the rat, supplied trace amounts of selenium was reported [Schwarz and Foltz, J. Am. Chem. Soc., 79, 3292 (1957)]. This report has stimulated the development of a large and growing body of work on the biological and nutritional roles of selenium and has led to the acceptance of selenium as an essential trace element. Recently, several selenoproteins, including selenoenzymes, have been reported [reviewed by Stadtman, Science, 183, 195 (1974)]. Developments in this area are being monitored and additional studies on the chemistry and biochemistry of selenium are anticipated.

Studies are in progress on various unresolved problems in chemotherapy, which hopefully will lead to the development of novel and superior chemotherapeutic agents.

Publications: None.

| SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space) | U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT** | PROJECT NUMBER Z01 AM 19806-02 LC |
|---|---|---|

PERIOD COVERED

July 1, 1976 to September 30, 1977

TITLE OF PROJECT (80 characters or less)

Nature of Steroid-Receptor Interactions

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: S. S. Simons, Jr.
Other: D. F. Johnson

COOPERATING UNITS (if any)

E. I. B. Thompson, NCI
J. V. Silverton, NHLBI

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

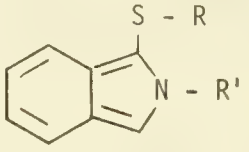
The objective of this project is to define the initial, intra-cellular events of steroid hormone action. Synthetic glucocorticoid derivatives, some of which could potentially react to form covalent receptor-steroid complexes, are being used with glucocorticoid-responsive rat hepatoma tissue culture cells to examine: (1) steroid-receptor binding site interactions; (2) the effects of steroid binding on receptor conformation; (3) the nature of "activation" of receptor-steroid complexes and (4) the nuclear binding of activated receptor-steroid complexes. Fluorescence spectroscopy represents a novel technique for examining these interactions. For this reason, we have investigated the fluorogenic reaction of o-phthalaldehyde and β-mercaptoethanol with primary amines and defined the fluorescent products as previously unknown, 1-alkylthio-2-alkylisoindoles. Further studies have been directed at adapting these isoindoles for a fluorescent study of steroid-receptor interactions.



PHS-6040
(Rev. 10-76)

Project Description: Steroid specific events are induced by nanomolar concentrations of hormone in responsive cells. Thus, extremely sensitive methods are required in order to monitor the steroid at physiological levels. Fluorescence spectroscopy can 1) detect some compounds at concentrations as low as $10^{-12}$ M and 2) provide information about the microenvironment of the fluorescent molecule. These two properties make fluorescence spectroscopy ideally suited for a study of steroid-receptor interactions.

We have examined the fluorogenic reaction of o-phthalaldehyde (OPTA) and β-mercaptoethanol (MERC) with primary amines and amino acids because the hitherto undefined, intensely fluorescent products are detectable at concentrations $\leq 10^{-9}$ M. We have determined the structure of these fluorescent products to be the previously unknown 1-alkylthio-2-alkylisoindoles I. While these compounds are unstable, we have also




I.

R = alkyl, aryl

R' = alkyl

succeeded in isolating the first stable member of this class of compounds (i.e., I where R = t-Bu, R' = n-Pr). The mechanism of the reaction, the nature of the intermediates, the scope and limitations of the reaction and the properties of the fluorescent products have all been examined. These studies have enabled us to modify the OPTA-MERC-amine reaction, and obtain a more versatile fluorescent isoindole. We are presently investigating the feasibility of attaching these isoindoles to a glucocorticoid steroid, which could permit a fluorescence study of steroid-receptor interactions.

Alternative methods of studying receptor-steroid interactions are 1) to examine the effects of subtle modifications of a steroid hormone and 2) to prepare steroids capable of forming a covalent receptor-steroid complex. Several 21-substituted derivatives of cortisol have been prepared; but, this project is only in the beginning stages. Numerous other derivatives, both of cortisol and dexamethasone, are scheduled to be synthesized during the next year.

The biological testing of the synthetic steroid derivatives is being done in collaboration with Dr. Thompson of the NCI. The various assays - both cell-free receptor binding studies and whole cell inducibility experiments using a line of rat hepatoma tissue culture cells - have been established and run with several cortisol derivatives; but, these studies are clearly just beginning. As more steroid derivatives become available, the activity in this phase of the project will increase. Eventually, when the synthetic work decreases, another line of hepatoma cells, grown without collaborative efforts, will be used to evaluate receptor homology.

Project No. ZO1 AM 19806-02 LC

We are applying organic chemistry to the study of an important biolcgical molecule(s) - the intracellular steroid hormone receptor(s). A molecular understanding of steroid-receptor interactions is highly desirable, especially since therapeutically valuable, steroid-specific differences are most likely expressed at this level of steroid hormone action.

Publications:

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Simons, S. S., Jr.: Factors influencing association of glucocorticoid receptor-steroid complexes with nuclei, chromatin and DNA: Interpretation of binding data. In Baxter, J. D. and Rousseau, G. G. (Eds.): Mechanisms of Glucocorticoid Hormone Action. Springer-Verlag, in press.

| SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space) | U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT | PROJECT NUMBER Z01 AM 20000-10 LC |
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PERIOD COVERED
July 1, 1976 through September 30, 1977

TITLE OF PROJECT (80 characters or less)
Pharmacodynamic Amines and Enzymes Involved in Their Metabolism

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: C. R. Creveling, Research Chemist, LC NIAMDD
OTHER: J. W. Daly, Chief, Section on Pharmacodynamics, LC NIAMDD

COOPERATING UNITS (if any) B. Hartman, Wash. U., St. Louis, MO., C. Isersky, NIAMDD CI; L. Tice, NIAMDD LEP; L. A. Sternberger, Edgewood Arsenal, MD.; B. Highman, Nat. Ctr. Toxicol. Res., Jefferson, AK.; R. Friedman, NIAMDD LC; R. T. Borchardt, U. Kansas, Lawrence, KA.; J. L. Costa, NIMH LCS; B. Nikodijevic, U. Skopje, Yugoslavia;

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Laboratory of Chemistry

SECTION
Section on Pharmacodynamics

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland, 20014

| TOTAL MANYEARS: | PROFESSIONAL: | OTHER: |
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CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The purpose of this project is to study the chemistry and biochemistry of biogenic amines, their amino acid precursors and transformation products. The areas of specific interest are:

1) The mechanism of the neurotoxicity of 6-hydroxydopamine, 5,7-dihydroxytryptamine and related compounds;
2) The role of adrenergic innervation in the release of calcitonin from the C-cell of the thyroid;
3) The effect of substitution of fluorine on the aromatic ring of dopamine, norepinephrine, and serotonin on transport mechanisms in vivo, in atrial preparation, brain synaptosomal preparation, and in platelet preparation;
4) The mechanism of toxicity and leukocytopoiesis of 2-fluorohistidine in normal and leukemic mice;
5) The metabolism and incorporation into protein of 2-fluorohistidine in mouse and murine erythroleukemia cell cultures;
6) The immunolocalization of catechol-O-methyltransferase at cellular and subcellular sites.



PHS-6040

A. Rotman, Hadassah Med. Sch., Jerusalem, Israel; L. I. Goldberg, U. Chicago, Chicago, IL.; J. Lundstrom, Astra Pharmaceuticals AB, Södertäje, Sweden; H. F. Woods, Univ. Sheffield, Sheffield, England; J. D. Kohli, Univ. Chicago, Chi., IL.; J. Tarver, Hoffman La Roche, Inc., Nutley, NJ.; R. H. Adamson, NCI ICHPH; W. F. Anderson, NHLBI, MH; B. Bernard, Univ. Conn., Storrs, CO.; J. Ludford, NIDA, BB; J. E. Mosiman, DCRT LSMM.

Project description:

Objectives: To study the basic chemistry and biochemistry of the catecholamines, phenolic amines, indole alkylamines, imidazolealkylamines, and their precursors and metabolic transformation products. To develop new techniques for the investigation of membrane transport systems and receptor interaction as related to these amines and their interaction with various drugs. To study the mechanism of action of specific neurodegenerative agents structurally related to these amines; to investigate the specific sites of the cytotoxic action of these compounds and to describe both the subcellular morphological changes and specific interaction with macromolecules which accompany this cytotoxic action. To study the histopathology of the non-neuronal effects of these cytotoxic amine derivatives. To isolate and purify enzymes operative in the biosynthesis, metabolism, and physiological function of biogenic amines. To determine the physical properties of these enzymes. To prepare specific antibodies for these enzymes and related protein factors and apply the antibodies to the development of radioimmunoassays for the measurement and localization of these proteins. To investigate the distribution of these enzymes and proteins at both the cellular and subcellular levels through the application of immunoperoxidase, ferritin and fluorescence techniques. To determine the effects of the introduction of fluorine and other substituents on the aromatic and imidazole ring of biogenic amines on transport, metabolic, and receptor-mediated systems for biogenic amines. To investigate the metabolism, toxic actions, and antineoplastic effects of various fluorine analogs of the amino acid precursors of certain biogenic amines and to ascertain the nature and mechanism of the incorporation of these analogs into protein in vivo and in various cultured cell lines. To devise and test a system for the quantitative measurement of rodent behavior and to correlate such measurements with function of biogenic amine systems in brain.

Methods Employed: Drugs, biogenic amines, and various precursors have been prepared with deuterium, iodine-125, carbon-14, and tritium to study the metabolism, transport, distribution, and release of these compounds. Standard methods of synthetic organic chemistry have been used to prepare these compounds. Standard methods of protein purification and analysis have been used to purify and isolate various enzymes and proteins, as well as specific affinity labeling techniques and immunoabsorption techniques. Standard methods of histopathology employing light and electronmicroscopy have been used to examine the cytotoxic effects of neurotoxic amines and certain cell specific toxic amino acid derivatives. Standard immunodiffusion, electrophoresis and immunization techniques have been employed in the development of various antibodies. Standard methods of cell culture have been used for the testing of various amino acid analogs on various cell lines. New techniques for the fixation of tissues to facilitate the localization of antibody-specific pro-

teins have been developed. A new system for the quantitative analysis of rodent behavior has been explored and has required the development, through a trial and error procedure of computer analysis to interpret the validity of the behavioral parameters.

Major Findings: Studies on the mechanism by which 6-hydroxydopamine and 5,6-dihydroxytryptamine and related compounds induce a specific neurotoxicity in vivo have been continued. An oxygen-dependent binding and subsequent cross-linking of model proteins was demonstrated. Stoichiometric measurements of this binding and other properties indicate that the binding and cross-linking occurred primarily through free sulfhydryl groups on protein. Attempts have been made to relate this model for cytotoxicity to events in neuroblastoma cultures, isolated mouse atrial preparations, mitochondrial preparations, and in intact animals. A rationale for the design of new cell-specific toxic agents was developed based on these studies. While the present results indicate a relationship between the irreversible binding of neurotoxic amines and the cytotoxic sequela, no direct evidence for amine-elicited polymerization in vivo has been obtained (Creveling).

The mechanism of cytotoxicity of 5,7-dihydroxytryptamine differs substantially from that of both 5,6-dihydroxytryptamine and 6-hydroxydopamine and its cogeners in that it fails to bind to model proteins in vitro and its action in vivo is blocked by prior treatment with monoamine oxidase inhibitors. Studies with isotopically labelled 5,7-dihydroxytryptamine both in vitro and in vivo indicated that the mechanism of its cytotoxic action could be subsequent to the enzymatic formation of an aldehyde intermediate (Creveling).

A non-neuronal, cytotoxic effect of 5,6-dihydroxytryptamine leads to the development of a time and dose-dependent development of subepicardial lesions of the myocardium. These lesions were preceded by a fatty infiltration of myocardial fibers. There was no apparent relationship between the specific neurotoxicity and the effects on the myocardium. Studies on the uptake and cellular and subcellular distribution of 5,6-dihydroxytryptamine indicated that there is a relatively large component of the uptake into non-neuronal tissue in heart preparations. The observed uptake was temperature-dependent and essentially irreversible. The nature and site of this binding phenomenon is under continuing investigation (Creveling).

Studies on the inhibition of norepinephrine uptake and the release of norepinephrine both in vivo and in isolated atrial slices are being continued to find additional compounds which may be useful as investigational tools. The kinetic parameters of the uptake mechanism are currently under study as a model for investigating the mechanism responsible for the transport of amine through the neuronal membrane. The transport and storage of the fluorotyramines, dopamines, and indoleamines in atrial preparations are being examined. The isolated atrial system in vitro is being used as a model to measure the release of amines stored intraneuronally. The possible involvement of S-adenosyl-L-methionine-dependent carboxymethyltransferase in the release of amines is being examined through the use of product inhibition of the transferase. (Creveling)

The cellular localization of catechol-O-methyltransferase was determine

through the application of immunoperoxidase techniques using an antisera specific for the soluble form of the enzyme from liver. The study revealed an important localization of the enzyme not only in liver and kidney tubules but also in the reproductive organs of both male and female rat. Further, the presence of the enzyme was described in the spleen, bone marrow, lymphatics, and thymus. The results suggest that catechol-O-methyltransferase is important in the inactivation of steroidal catechols (Creveling).

A study of the kinetics of the inhibition of catechol-O-methyltransferase by a specific antibody preparation for this enzyme was carried out. This study indicated that the antigenic sites differed from the catalytic sites on the enzyme molecule (Creveling).

A study of the cellular localization of catechol-O-methyltransferase in rat brain using an antibody for the soluble form of the enzyme isolated from rat liver by an immunofluorescent technique revealed the pressure of "glial"-like, enzyme-positive cells and a fine network of what appear to be dendritic processes (Creveling).

The intracellular localization of catechol-O-methyltransferase is being investigated with immunological techniques. The results indicate that the enzyme is present on rough coated particles, reticular endothelial structures, and perhaps plasma membranes. However, technical difficulties make a definitive localization at the ultrastructural level impossible at present (Creveling).

A study of the toxicity and histopathology of 2-fluorohistidine have demonstrated an interesting, apparently specific effect of this analog of histidine upon blood forming organs. A non-lethal dose of 2-fluorohistidine results in a time and dose dependent leucopenia, regression of the spleen, thymus, lymph nodes, and a profound hypocellularity of bone marrow. This effect has been explored as a therapeutic tool in leukemic mice with some success. The specific nature of the 2-fluorohistidine-effect is being investigated in various leukemia models in vivo, in cultured murine leukemia cells, and in normal mice (Creveling).

Studies on the metabolism of 2-fluorohistidine and a comparison to the metabolism of histidine are in progress. The present evidence indicates that 2-fluorohistidine substitutes in a competitive fashion with histidine for histidine amino acyl transferase resulting in the incorporation of 2-fluorohistidine into protein. 2-Fluorohistidine is a substrate for histidine ammonia lyase and gives raise to 2-fluorourocanic acid. The further metabolism is at present unknown but the present evidence suggests a direct entrance into tetrahydofolic acid metabolism (Creveling).

A collaborative project with NIDA to develop a new technique for the evaluation of animal behavior has been explored. The system involves the point to point tracking of one to six animals in an open field. The location of each animal, via a color television camera, is determined 10 times a second and information recorded and formated by an on-line computor onto a high speed tape. Software systems have been developed to analyze the motion patterns. This system is without precedent, necessitating a trial and error procedure for evaluating the significance of the selected patterns. At present,

a set of thirty parameters which are designed to compare the interaction of two mice appears to be a promising avenue of approach. Studies were carried out with a variety of conditions to test the system. These included eight strains of mice, male-female comparisons, environmentally "deprived" vs environmentally enriched rodents, mice treated at birth with 6-hydroxydopamine and compared with untreated controls over a period of 8 months, animals treated with a variety of psychoactive drugs, and animals with specific amine acid deficiencies. These studies were done simultaneously with studies on amine-sensitive cyclic AMP-generating systems in rodent cortex (Creveling, Daly).

Significance to Biomedical Research and the Program of the Institute: The biosynthesis, uptake, and metabolism of biogenic amines and their amino acid precursors are of fundamental importance in neurochemistry and pharmacology and have direct bearing on the function of both the central and peripheral nervous systems. The role of catechol-O-methyltransferase is important with regard to the functioning of the cardiovascular system, the detoxification of xenobiotic catechols, the inactivation of catechol steroids and in the metabolism of catecholamine transmitters in the central nervous system. An understanding of the role, localization, etc. of this enzyme may provide insight into a variety of pathological conditions in man, including essential hypertension. An understanding of the mechanism of action of various cytotoxic amines will not only provide a rationale for the design of additional specific cytotoxic agents, but may provide guide lines for the application of such agents toward other cell types including neoplastic cells. The study of analogs of both amines and their amino acid precursors have provided new compounds of great interest (2-fluorohistidine) as biochemical tools and potential effectiveness as antineoplastic and immunosuppressive agents.

Proposed Course of the Project: The mechanism of the cytotoxic action of various amines will be studied to ascertain the specific molecular structure of the interaction products of the amines with protein. Additional systems will be investigated to attempt to demonstrate that the binding and cross-linking phenomena are operative in vivo. The metabolism of 2-fluorohistidine will be investigated in mouse and MEL cell cultures with regard to the formation of metabolites analogous to those derived from histidine. Attempts will be made to isolate and analyze proteins in which 2-fluorohistidine is a component to directly determine if this analog can replace histidine in the peptide structure. The course of p388 leukemia in mouse will be compared to the course of the disease under 2-fluorohistidine therapy. The synergism between 2-fluorohistidine and histidine and the toxicity of the fluorine analog will be explored. The exploration of the specific cellular location of catechol-O-methyltransferase in the central nervous system will continue as will attempts to determine the subcellular locus of this enzyme. Similar studies are being initiated with antibodies to phosphodiesterases and certain heat stable activator proteins for brain phosphodiesterases. The study of the uptake and release of norepinephrine and related compounds by atrial preparations and brain synaptosomal preparations will continue. The interaction of fluorine derivatives of the catecholamines, indolamines, and imidazoleamines with neuronal transport systems, uptake systems present in platelets, and in synaptosomal systems will be continued. A verification of the first phase of the animal behavior monitoring system will be concluded and a modification of the

software systems to permit direct interaction of the experimenter with the system developed.

Publications:

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| SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space) | U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT | PROJECT NUMBER ZO1 AM 20001-07 LC |
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PERIOD COVERED
July 1, 1976 through September 30, 1977

TITLE OF PROJECT (80 characters or less)
The Role of Cyclic Nucleotides in the Nervous System

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: J. W. Daly, Chief, Section on Pharmacodynamics, LC, NIAMDD

OTHER: C. R. Creveling, Research Chemist, LC, NIAMDD
P. Skolnick, Senior Staff Fellow, LC, NIAMDD
C. Davis, Staff Fellow, LC, NIAMDD
T. Kanamori, Visiting Fellow, LC, NIAMDD

COOPERATING UNITS (if any) Y. Nimitkitpaisan, Guest Worker, Morgan State University; D. S. Segal, Psychiatry Dept., Univ. Calif. San Diego, LaJolla, Calif.; J. N. Davis, Duke Univ. Durham, N.C.; D. Taylor, Lab. Neuropharmacology, NIMH, St. Elizabeths, Wash., D.C.; S. Paul, Lab. Clinical Science, NIMH

LAB/BRANCH
Laboratory of Chemistry

SECTION
Section on Pharmacodynamics

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland, 20014

| TOTAL MANYEARS: | PROFESSIONAL: | OTHER: |
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☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The role of cyclic nucleotide-generating systems to the functional operation of the central nervous system is being investigated from a number of standpoints: i) Alterations in the levels or properties of brain adenylate cyclases, phosphodiesterases and protein kinases as a result of pretreatments of animals with drugs, hormones, or environmental manipulation. ii) The nature and homeostatic control of cyclic AMP and cyclic GMP-generating systems in brain slices with emphasis on the types of neurotransmitters and antagonists which interact with the systems, iii) Correlation of effects of drugs on the function of cyclic AMP and cyclic GMP-systems in brain slices and cell-free preparations with in vivo effects of the drugs on behavior and vegetative function. iv) Strain and species differences in the magnitude and character of responses of cyclic AMP-generating systems in brain slices to catecholamines, serotonin, histamine, adenosine, prostaglandin, and depolarization and the sensitivity of such animals to drugs affecting these neurotransmitter substances. v) The nature and control of the interactions of ions such as calcium, magnesium, manganese, and zinc with the cyclic nucleotide-generating systems of brain.

Project Description: Project No. ZO1 AM 20001-07 LC

Objectives: To determine the factors that govern the formation and metabolism of cyclic AMP and cyclic GMP in the central nervous system. To investigate the possible role of cyclic nucleotides in regulating biochemical reactions and membrane properties in brain tissue. To investigate the interrelationship between central neurotransmitter function and cyclic nucleotides. To investigate the role of adaptive changes in cyclic nucleotide-generating systems in drug-tolerance and dependence.

Methods employed: Surgical techniques, environmental manipulation, drug-regimens have been used to alter central function. Formation and metabolism of cyclic nucleotides in biological preparations have been studied with isotopically labeled compounds.

Major findings: The antagonist activity of clonidine and various analogs at norepinephrine-sensitive cyclic AMP-systems in rat brain slices showed no correlation with their activity as α-agonists in peripheral systems. Clonidine antagonized amphetamine-excitation in rats and it was proposed the antagonism of α-adrenergic agonist-sensitive cyclic AMP-generating systems by clonidine and phentolamine provides a possible basis for the similar central depressant activity of these two compounds which are normally considered an α-agonist and an α-antagonist respectively (Skolnick).

The effect of various agonists and antagonists on α-adrenergic agonist-sensitive adenylate cyclase in rat brain slices was shown to correlate with their effects on binding at the α-antagonist, WB 4101, to rat brain membranes. It was proposed that this compound is a useful tool for the study of α-sensitive cyclic AMP-systems in brain (Skolnick).

Tazolol, a reported β-agonist in heart, was shown to be a potent β-antagonist of norepinephrine-elicited accumulations of cyclic AMP in rat brain slices and a potent inhibitor of the depressant effects of norepinephrine on firing of central neurons (Skolnick).

The denervation-induced hyperresponsiveness of norepinephrine-sensitive cyclic AMP-generating systems in brain has been investigated in detail with two strains of rats. 6-Hydroxydopamine was used to disrupt noradrenergic input. In Sprague-Dawley rats both α- and β-responses were enhanced by about 2 fold after 6-hydroxydopamine, while in Fisher 344 rats, the noradrenergic responses which were already relatively high in control animals were not further increased by 6-hydroxydopamine-treatment. Binding at a β-adrenergic ligand, dihydroalprenolol was enhanced by about 25% after 6-hydroxydopamine in both rat strains. Binding of an α-adrenergic ligand, WB 4101, was unchanged. Thus, levels of receptors do not correlate with changes in responsiveness of cyclic AMP-systems. Instead, data with homogenates suggest that alterations in adenylate cyclase activity may account for the adaptive changes in Sprague-Dawley rats after 6-hydroxydopamine (Skolnick).

Estrogen-elicited accumulations of cyclic AMP in incubated intact hypothalmi had, in earlier studies, been shown, based on antagonist paradigms, to be mediated by catecholamines. Further support for catecholamine as intermediates was provided by data demonstrating that turnover of catecholamines is

enhanced after prolonged incubation of hypothalami with diethylstilbestrol. Catechol estrogens were found to inhibit estrogen-elicited accumulations of cyclic AMP in hypothalami of female rats, apparently by competition at an estrogen binding site. The results strongly suggest that 2-hydroxyestradiol is an endogenous antiestrogen (Skolnick).

Histamine-elicited accumulations of cyclic AMP in guinea pig cerebral cortical slices involved both $H_1$- and $H_2$-histaminergic receptors. The $H_1$-response was greatly potentiated by adenosine and was dependent on the presence of extracellular calcium ions. These results parallel prior results with norepinephrine-sensitive cyclic AMP-generating systems in brain slices where the α-response was found to be potentiated by adenosine and to be dependent on extracellular calcium. A vesicular or "microsac" preparation from rodent brain showed both $H_1$ and $H_2$-responses similar to those of brain slices and is being further investigated (Creveling).

During isolation of calcium-dependent activator protein from rat brain, a number of heat-stabile inhibitors and activators of phosphodiesterases were isolated. At least three factors were present which inhibited the calcium-dependent phosphodiesterase. Two were low molecular weight protease-insensitive compounds. These low molecular weight compounds appeared to be potent inhibitors for phosphodiesterases in a "microsac" preparation from brain. At least two protein factors were present which inhibited calcium-independent phosphodiesterase. At least four protein factors were present which activated high Km cyclic AMP-phosphodiesterase (Kanamori).

Calcium-dependent activator protein was found in brain slices to be present both membrane-bound, presumably associated with adenylate cyclase, and in the cytosol, presumably associated with phosphodiesterase. The effects of stimulation of cyclic AMP-generating systems on the subcellular distribution of activator protein in brain slices is under investigation (Davis).

Deoxycoformycin and erythro-9-(2-hydroxy-3-nonyl)adenine have been shown to be potent inhibitors of rat brain adenosine deaminase. The deoxycoformycin potentiates amine-elicited accumulations of cyclic AMP in rat brain slices, apparently by protecting endogenous adenosine from enzymatic deamination (Nimitkitpaisan).

Significance to biomedical research and the program of the Institute: The key role of cyclases and cyclic AMP and cyclic GMP in regulating cellular activity in response to external stimuli in many biological systems make elucidation of their role in brain of fundamental importance to an understanding of function in this organ.

Proposed course of project: The morphological location of the labeled pools of adenine nucleotides will be determined. The role of phosphoribosyl transferase and adenosine kinase in maintaining this pool will be explored. Secondary effects on enzymes and modulators due to elevated cyclic AMP levels in brain tissue will be investigated. The *in vivo* effects of drugs and the effects of lesions and hormonal alterations on the responsiveness of the cyclic AMP-systems will be further explored. The nature of the adenosine-receptor will

be studied further using adenosine analogs and antagonists modified for possible affinity labeling. Other agents with selective effects on adrenergic, serotonergic, and histaminergic systems will be sought. The role of calcium and other ions in the function of cyclic AMP-generating systems will be further investigated, both in vivo and in vitro. Correlations between cyclase responses and behavorial activity will be studied in crosses between various rodent strains. The nature of the GTP-binding site on adenylate cyclase will be investigated. Correlations between cyclic GMP and cyclic AMP levels in brain slices will be attempted. Antibodies to enzymes and regulatory modulator proteins involved in the cyclic AMP-generating systems will be prepared and effects of various regimens on the turnover, levels, and distribution of these enzymes and regulatory proteins in brain will be investigated. Further attempts to develop more satisfactory inhibitors of cyclases, phosphodiesterases, and kinases will be made.

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guinea pig cerebellar slices. Biochim. Biophys. Acta, in press.

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| SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space) | U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT | PROJECT NUMBER Z01 AM 20200-09 LC |
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PERIOD COVERED
July 1, 1976 to September 30, 1977

TITLE OF PROJECT (80 characters or less)
Enzymatic Oxidation of Drugs to Toxic and Carcinogenic Metabolites

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

| | | | | |
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| | D. R. Thakker | Staff Fellow | LC | NIAMDD |
| | H. D. Mah | Guest Worker | LC | NIAMDD |
| | R. E. Lehr | Guest Worker | LC | NIAMDD |
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COOPERATING UNITS (if any)
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Laboratory of Chemistry

SECTION
Section on Oxidation Mechanisms

INSTITUTE AND LOCATION
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CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The primary goal of this project has been the elucidation of the reactive metabolites which are responsible for the carcinogenic, cytotoxic, and mutagenic activity of benzo[a]pyrene and other polycyclic hydrocarbons. The approach taken consists of: i) synthesis of primary oxidative metabolites as well as selected secondary oxidative metabolites, ii) study of the metabolism of these hydrocarbons with liver microsomes, as well as purified and reconstituted cytochrome P-450 systems with and without epoxide hydrase, iii) tests for inherent mutagenicity of the synthetic metabolites toward bacterial and mammalian cells, vi) elucidation of the roles of the cytochrome P-450 system and epoxide hydrase in potentiating or obliterating the mutagenicity of these metabolites, v) determination of the carcinogenic activity in vivo for these compounds, vi) determination of the rate and nature of the products formed when reactive metabolites such as arene oxides and diol epoxides react with biopolymers and less complex chemical analogs. Attempts to formulate a general theory of hydrocarbon-induced carcinogenesis are in progress.



PHS-6040
(Rev. 10-76)

Project Description:

Objectives: To study the basic mechanisms of enzymatic oxygen activation and incorporation into organic substrates. To explore enzymatic and nonenzymatic reactions of labile intermediates such as epoxides and peroxides. To devise methods for the synthesis and study of the chemical reactions of arene oxides. To investigate the role of labile oxygenated intermediates in the cytotoxicity and carcinogenicity of xenobiotic substances.

Methods Employed: Isotopically labeled substrates, water, and oxygen gas have been used to study the oxidation of organic compounds to labile metabolites by a variety of enzymes. Nuclear magnetic resonance spectroscopy, mass spectrometry, x-ray crystallography and other physico-chemical techniques have been employed frequently in these studies. In addition to the usual techniques employed for the separation of metabolites, high pressure liquid chromatography is being pioneered in this area. Mutagenesis is studied in bacterial and mammalian cells and correlated with whole animal carcinogenicity.

Major Findings: Previous annual reports from this Section have described a systematic approach to the study of the cytotoxicity, mutagenicity, and carcinogenicity of the ubiquitous environmental carcinogen benzo[a]pyrene (BP). Briefly these studies have consisted of i) synthesis of as many known and potential oxidative metabolites as was possible, ii) study of the metabolism of the hydrocarbon with these authentic standards in hand, iii) testing of these compounds for cytotoxic and mutagenic activity with bacterial and mammalian cells both in the presence and in the absence of added drug metabolizing systems such as cytochrome P-450 and epoxide hydrase, and iv) evaluation of the carcinogenicity of these synthetic standards in several animal models. The majority of these studies on benzo[a]pyrene are nearing completion.

Studies of the inherent mutagenic and cytotoxic activity of all twelve isomeric phenols, four of the six metabolically possible arene oxides and corresponding trans-dihydrodiols, and five quinones of BP have been examined in histidine dependent mutants of Salmonella typhimurium and 8-azaquanine sensitive Chinese hamster V-79 cells. The most mutagenic of these compounds was BP 4,5-oxide, although several of the phenols had weak but significant mutagenic activity. The phenols, dihydrodiols, and BP were subjected to metabolic activation with a cytochrome P-448 containing system in the presence of the bacteria with the result that BP 7,8-dihydrodiol was more strongly activated to mutagens than were the other compounds. This mutagenic activity was presumed due to the diastereomeric 9,10-oxides of the 7,8-diol. The mutagenic activity of these 7,8-diol-9,10-epoxides was not blocked by the presence of epoxide hydrase and the presence of the hydroxyl groups at the 7- and 8-positions was found to be nonessential to mutagenic activity. The 7,8-diol-9,10-epoxides are among the most mutagenic compounds yet tested in V-79 cells. With the conditions for metabolic activation established in the experiments with the BP derivatives, the five metabolically possible trans-dihydrodiols of benzo[a]anthracene (BA) were then examined. As anticipated from quantum mechanical calculations, the BA 3,4-dihydrodiol caused the

highest number of mutations. This result provides the basis for a new theory of hydrocarbon-carcinogenicity which will be described later.

In the previous annual report, BP 7,8-oxide was described as strongly carcinogenic on mouse skin but weaker than BP. Study of the 4-, 5-, 6-, 7-, 8-, 9-, and 10-hydroxy BP on mouse skin has shown these compounds to be non-tumorgenic under conditions where BP caused tumors in all the animals. These studies show that the carcinogenicity of BP 7,8-oxide is not due to its phenolic isomerization products and casts serious doubt on the theory that 6-hydroxy BP is an ultimate carcinogen from BP. BP 7,8-dihydrodiol which results from the action of epoxide hydrase on BP 7,8-oxide is a better skin carcinogen than BP 7,8-oxide and is equipotent to BP. However, in the newborn mouse, BP 7,8-dihydrodiol is much more tumorgenic than BP and can be considered as a proximate carcinogen. On mouse skin, the 7,8-diol-9,10-epoxides and 9-hydroxy BP are potent agents in causing hyperplasia.

Convenient synthetic methods have been devised for the preparation of specifically tritiated arene oxides such that these compounds could be examined as substrates for the glutathione transferases and epoxide hydrase. The most rewarding of the enzymatic studies have been those with epoxide hydrase in which a highly sensitive and rapid thin layer chromatographic assay was developed which showed that phenanthrene 9,10-oxide was one of the best known substrates for the enzyme. In addition, BP 4,5-oxide was found to be the most sensitive substrate for assaying small amounts of activity. Studies with optically active cis-stilbene oxides established that the enzyme has a marked preference for attack at an S-center in epoxide substrates. Studies with human livers established that the substrate requirements of both the human and rodent derived enzyme are very similar.

Studies of the metabolism of BP by microsomes from the lungs of inbred strains of mice which vary markedly in their susceptibility to chemical carcinogens showed very little in the way of differences in the profile of metabolites. There were, however, markedly different levels of control and carcinogen-induced activity for these strains. Examination of the metabolism of BP 7,8-dihydrodiol by liver microsomes and reconstituted drug metabolizing systems has shown dramatic stereospecificity and regiospecificity. In all cases, the dihydrodiol is converted into a mixture of the two diastereomeric 7,8-diol-9,10-epoxides. The (-)-enantiomer of the dihydrodiol is converted predominantly into one diastereomer while the (+)-enantiomer is converted preponderantly into the other diastereomer. Enzyme preparations from the livers of non-induced animals also form large amounts of 6-hydroxylated dihydrodiol. Studies of the combined stereospecificity of the cytochrome P-450 system and epoxide hydrase have indicated that (-)-BP 7,8-dihydrodiol is the predominant enantiomer formed from BP. This specificity is due to stereospecificity by the cytochrome P-450 system rather than epoxide hydrase. This result is of key significance to further carcinogenesis testing.

Since a mounting body of evidence has indicated that the 7,8-diol-9,10-epoxides of BP are ultimate carcinogens and since their in vivo formation occurs with enantiomeric specificity, the two pairs of enantiomeric diastereomers

have been synthesized, and their absolute stereochemistry has been assigned by optical methods. Comparison of the products formed between these four compounds and poly(G) in vitro with the RNA-hydrocarbon adducts formed when BP is applied to mouse skin have shown that one enantiomer of each diastereomer is bound to RNA in vivo. These diol epoxide adducts are derived from both enantiomers of BP 7,8-dihydrodiol. Chemically, these RNA adducts are formed by attack of the N-2 amino group of guanine at C-10 of the diol epoxides. Whether or not this structural modification of RNA or a similar one in DNA plays a role in carcinogenesis remains to be examined. Both of the diol epoxide diastereomers bind to the phosphate backbone in nucleic acid and thereby induce strand breaks.

In an attempt to generalize the concept of diol epoxides as ultimate carcinogens from polycyclic aromatic hydrocarbons, we have conducted quantum mechanical calculations which should predict reactivity for these compounds. These calculations predict which of the several diol epoxides of a given hydrocarbon should be most reactive and allow the hydrocarbons to be ranked for carcinogenicity based on the most reactive diol epoxide. In essence, these calculations require that the epoxide form part of a bay-region of the hydrocarbon (typified by the 4,5-region of phenanthrene) and provide a reasonable ranking of the hydrocarbons for carcinogenic activity. In order to test the bay-region theory, the five dihydrodiols of BA were synthesized and tested for mutagenic activity on metabolic activation. As predicted, the 3,4-dihydrodiol, which can be converted into a 1,2-epoxide (bay-region), was the most active. Diol epoxides of the BA dihydrodiols have been synthesized to expedite further mutagenesis testing.

Significance to Biomedical Research and the Program of the Institute: Although enzymatic oxidation and drug metabolism had long been held to constitute a means of detoxication, results of the type described here make it quite clear that certain of these pathways for drugs and environmental chemicals result in toxic and carcinogenic metabolites. Once the nature of these active metabolites have been identified, it may become clear as to which industrial and agricultural chemicals are unsafe for use. The potential impact on the design of safe drugs is clear. Clearly, a workable theory of hydrocarbon-induced carcinogenicity with predictive properties is of immense practical value.

Proposed Course of Project: The initial success of the "Bay-Region Theory" demands that this potentially powerful concept be examined in the fullest. To this end, we propose the synthesis of dihydrodiols from other carcinogenic and noncarcinogenic hydrocarbons, for this purpose. Carcinogenesis testing and metabolism studies of BP and its derivatives will be continued. The mechanism of action of the cytochrome P-450 system and epoxide hydrase will be further explored.

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ANNUAL REPORT
LABORATORY OF EXPERIMENTAL PATHOLOGY

Studies in the Section of Molecular Pathology (Dr. George G. Glenner and staff) have continued to be concerned with various aspects of the pathogenesis of Amyloidosis.

Origin and Structure of Human Amyloid Proteins: Further studies on amyloid proteins and their origin have utilized a mono-specific anti-AA antibody to demonstrate in tissues by indirect immunfluorescence the site of the AA protein, usually associated with the amyloid fibrils of secondary amyloidosis and reported as a major constituent of the fibrils in a case of familial Mediterranean fever. The antibody stained secondary amyloid deposits as well as intracytoplasmic material in reactive lymphoid tissues. No staining was observed when control antisera were used. AA containing cells were most frequently observed in reactive lymphoid tissues. Tonsils from patients with chronic tonsillitis invariably contained large numbers of positive cells. Spleens showing reactive lymphoid hyperplasia were also frequently positive. Conditions in which the AA positive cells were especially numerous included chronic infections, Sprue and Feltys' syndrome. Fewer lymph nodes in which reactive hyperplasia was prominent were studied, but in two such examples, the cells had characteristics of plasma cells. Their distribution in lymphoid tissue was also like that of plasma cells. In tonsils, they were found in subepithelial and interfollicular zones, in lymph nodes, in the medulla; and in spleens, in the marginal zone and red pulp. Approximately 10-20% of AA-containing cells also contained IgG. These data suggest that the AA protein and immunoglobulin usually are not synthesized or secreted simultaneously. Further studies, perhaps at the electron microscopic level, are needed to demonstrate whether or not AA-containing plasmacytoid cells are in fact plasma cells, and not cells of an independent immunocompetent line. The question of synthesis versus processing or selective membrane reception must also be considered. The appearance of AA-containing cells in the liver was different from those in lymphoid tissues. In liver the cells apparently contained the protein. This could be a consequence of non-specific phagocytosis or the protein could be processed and/or secreted by these cells. However, phagocytic cells in lymph nodes and spleens did not appear positive. (Dr. Glenner)

Origin and Structure of an Acute Phase Protein (SAA) Antigenically Related to an Amyloid Fibril Protein (AA): Amyloidosis is a group of diseases in which insoluble protein fibrils having a beta pleated sheet conformation accumulate in tissues. Amyloid fibril proteins are thought to be derived from normally innocuous polypeptide precursors by proteolysis or other physiochemical mechanisms. The major fibril protein isolated from human secondary amyloid deposits and from most spontaneous and induced amyloid deposits in experimental animals has been designated AA. Antibodies to denatured AA polypeptides cross-react with a serum protein which has been designated SAA.

Isolation of SAA. The first project, undertaken in 1973, was to purify SAA from human serum by affinity chromatography using anti-AA immunosorbents.

These endeavors were unsuccessful because SAA was not retained by the affinity column; this observation was quite puzzling in view of the fact that anti-AA and SAA-containing serum formed strong precipitin lines in Ouchterlony double immunodiffusion. Earlier attempts by Dr. Linke to isolate SAA by conventional size and charge fractionation techniques and by differential precipitation had been unsuccessful because SAA was invariably "lost" after purification, according to Ouchterlony analysis of serum fractions. Dr. Sipe recognized the need for a quantitative assay to monitor the isolation of SAA and to study its origin and structure. In collaboration with Dr. Linke an antigenic immunosorbent column was prepared from a highly purified AA preparation of homogenous size and charge and known primary structure at the amino terminus. Antibodies purified from antiserum raised to crude denatured AA fibrils were employed by Dr. Linke to monitor the isolation of SAA and by Dr. Watanabe to investigate the cellular distribution and possibly the cellular origin of the AA protein by indirect immunofluorescence. Dr. Sipe, in collaboration with Dr. Ignaczak, developed a solid phase radioimmunoassay for the AA protein which employed purified antibodies to purified AA protein. These antibodies were adsorbed to the wells of flexible microtitration plates and cross-reacting antigens in serum and tissues were determined according to their degree of competition with $^{125}$I-AA. When this quantitative assay was used to monitor the isolation of SAA several interesting observations on the structure and properties of SAA were made.

Structure of SAA: The molecular weight of native SAA was determined by the Ouchterlony technique to be 160,000-200,000 and that of guanidine HCl denatured SAA (designated SAAL) to be 12,500. When Dr. Sipe analyzed by RIA the gel filtration of SAA in guanidine HCl, it was found that 90% of SAA was not dissociated to SAAL, but had remained undetected by double immunodiffusion with anti-AA antibodies. Formic acid denaturation of SAA-containing serum had been shown by other laboratories to be effective in forming SAAL, and we determined by RIA that 85% of SAA was dissociated to SAAL by this agent.

The above work established that SAA consisted of SAAL complexed either with itself or other polypeptides by noncovalent bonds. One experimental approach to determine whether there was a high molecular weight polypeptide carrier for the SAAL polypeptide, was to radiolabel the SAA fraction from whole serum with iodine 125 and to then mix it with unlabeled, whole, SAA-containing serum and carry out immunoprecipitation with anti-AA antibodies using a double antibody technique. The immunoprecipitated $^{125}$I-SAA was analyzed by SDS urea-polyacrylamide gel electrophoresis and only a fraction of the precipitated radioactivity was observed to migrate with a molecular weight of 12,500. Multiple radiolabeled species and molecular weights intermediate to SAA and SAAL were also detected and appear to represent incompletely dissociated SAA, suggesting that SAA is an aggregate of several SAAL chains.

Certain interesting conformational properties of SAA were elucidated by the RIA analysis of SAAL isolation. It appears that not all determinants which cross-react with antibodies to the AA polypeptide are accessible in native SAA, and that they are exposed during the isolation of SAAL. This phenomenon is not solely due to the formation of a smaller cross-reacting

antigen, since formic acid derived SAAL is almost 50 fold more immunoreactive than SAAL prepared by guanidine HCl denaturation. The increase in immunoreactivity of SAA-containing serum was observed to a lesser extent upon storage of serum or experimental manipulation which does not normally dissociate noncovelent bonds.

The sensitivity of RIA for the detection of SAA was lower than expected when AA cross-reactivity values for sera were correlated with their reaction with anti-AA anti-AA antibodies in immunodiffusion. RIA determination of SAA in undenatured whole serum reaches maximum values of the order of 5 µg/ml, yet sera with values as low as 50 ng/ml were strongly reactive in double immunodiffusion. It appears that unfolding of cross-reacting determinants occurs during the process of double immunodiffusion in agarose gel, and this is additional evidence for the "buried" nature of AA determinants in native SAA.

Additional evidence for the conformational flexibility of SAA includes the observation that heterogeneous molecular weight species of SAA are formed during storage of serum at 4°C. This dissociation and aggregation is accompanied by an increase in immunoreactivity of a constant volume of serum. Because this conformational flexibility may play an important role in the induction of the beta pleated sheet conformation prior to fibril deposition in tissues (if SAA is the circulating precursor of the AA fibril protein) we compared SAA from patients with and without secondary amyloidosis and found that both types exhibit this flexibility in conformation. However, there are differences in susceptibility of the two types of SAA to SAAL formation. SAA from patients with secondary amyloidosis was less readily dissociated to SAAL than was SAA from patients without amyloidosis. Furthermore, isolated SAAL preparations from patients with amyloidosis, although similar in immunoreactivity to that from patients without amyloidosis, have a markedly greater tendency toward reaggregation even in the presence of formic acid. It seems highly significant that SAAL aggregates easily, and is then difficult to dissociate when it has a conformation such that antigenic determinants which cross-react with antibodies raised to the insoluble fibrils deposited in tissues are maximally exposed.

The increase in immunoreactivity of SAA-containing serum which accompanys the formic acid dissociation of SAA to SAAL is time and temperature dependent and is also affected by the concentration of serum in formic acid. Because reproducible inhibition curves can be obtained for a given serum sample after formic acid denaturation, this treatment is routinely employed now for analysis of all serum and tissue samples. (Dr. Sipe)

Acute phase nature of SAA. The relative occurence of SAA in serum samples from 500 individuals either healthy or in various disease states, was compar by RIA and double immunodiffusion analysis. SAA levels were elevated above normal in several categories of neoplastic, inflammatory and infectious diseases as well as in secondary amyloidodis. The lowest median value, 8 ng/nl, was observed for approximately 150 normal sera, with no age related increase in subjects ranging in age from 16 to 70 years. The non-Gaussian distribution of values is consistent with observations in other laboratories that SAA is an acute phase reactant in man; hence the RIA for SAA has no

prognostic or diagnostic significance for secondary amyloidosis.

Recent collaborative studies with Drs. McAdam, Elin, and Wolff have shown that acute phase SAA elevations can be produced in previously asymptomatic normal individuals by the nonantigenic pyrogen etiocholanolone, and that the acute SAA response exactly coincides with the acute phase C-reactive protein elevation which has long been used as a clinical marker for inflammation.

Dr. Sipe in collaboration with Dr. McAdam established a RIA for mouse AA similar to the one which was employed for human AA. They established that SAA is an acute phase reactant in the mouse and that this response can be induced by many antigenic and inflammatory stimuli. The only example we have found where SAA does not promptly rise after an acute phase stimulus is the C3H/HeJ strain of mice, which is unresponsive to the physiological effects of purified LPS. C3H/HeJ mice do respond to other acute phase stimuli with SAA elevation, as does the A/J strain which is resistant to induction of amyloidosis; thus production of SAA appears to be a normal phenomenon which occasionally may be involved with the development of a pathological state, amyloidosis. (Dr. Sipe)

Kinetic analysis of proteins related to the amyloid fibril protein AA during development of amyloidosis: The origin of AA and AA-related proteins during induction of amyloidosis in C3H mice has been investigated using the RIA for murine AA. The response of the amyloid resistant A/J strain of mice has been monitored to serve as a control for the non-amyloidogenic effects of the amyloid inducing agent, complete Freund's adjuvant with added Mycobacterium butyricum. The primary or preamyloid phase in C3H mice is characterized by an acute phase response to the amyloid-inducing agent in which the concentration of SAA is increased several hundred fold, and by a lag period in which SAA concentration is diminished as compared with acute phase levels, but still significantly elevated with respect to baseline concentrations. The profile of SAA concentrations in A/J mice after treatment with the amyloid-inducing agents is similar to that of the C3H mice in which amyloidosis is induced.

The secondary, or amyloidotic, phase in C3H mice is marked by a thousand fold increase in the concentration of AA-related protein in the spleen. AA proteins are found in the spleens of A/J mice much later, and then only in those instances where amyloidosis occurs. These findings are consistent with earlier morphological observations of many investigators which have given rise to the concept of the biphasic development of amyloidosis. The data also suggest that in the spleen, where the first amyloid deposits are detected, fibrils result from local cellular secretion. They further raise the question of whether SAA is actually the precursor of the AA fibril protein or is instead related to the polypeptide precursor of the AA protein through a common gene product. (Dr. Sipe)

Origin of SAA: The AA cross-reactivity in ng/mg wet weight of tissue was determined for liver, spleen, kidney, peritoneum, heart, lung, skin, fat, muscle, and whole animal homogenates during the early hours of the acute phase response to LPS. It was found that liver showed the earliest and

largest rise in antigen content of all tissues. This elevation was parallel to that of plasma, and was significantly greater than that of spleen. The liver has been well documented as the tissue of C-reactive protein origin. The identical time response of these two acute phase reactants (CRP and SAA) in response to an inflammatory agent would suggest that there is a common method of induction for the two proteins and that SAA arises from de novo protein synthesis rather than from breakdown of connective tissue, which has been postulated by other workers. (Dr. Sipe)

Primary structure of proteins related to AA and non-AA amyloid proteins: It is not known what factors dispose a small percentage of patients with acute recurrent and chronic inflammatory to develop amyloidosis, since, as was mentioned above, all of them can be expected to produce elevated concentrations of SAA, and extremely high SAA concentrations can be induced in previously asymptomatic individuals. It may be that the primary structure of SAA in amyloidotic individuals predisposes it to formation of beta pleated sheet fibrils upon proteolysis. Other factors such as a defect in the lysosomal processing of SAA or synthesis of the AA precursor by an abnormal cell or tissue type may be involved. SAAL has molecular weight of 12,500 and would be expected to contain approximately 30 additional amino acid residues compared with the AA fibril protein which has molecular weights of 5500 to 9000. Other laboratories have shown that SAAL has the same primary structure at the amino terminus as AA and hence the additional amino acids would be expected to comprise an additional peptide segment at the carboxyl terminus.

In collaboration with Dr. Waxdal, Dr. Sipe developed microsequence methodology for amyloid proteins using the Beckman spinning cup protein sequencer and radioactive PITC. This methodology has been applied to the amyloid fibril protein associated with the familial Portuguese amyloid neuorpathy which is very closly related to normal serum prealbumin (Collaboration with Drs. Costa and Sobiech). The preliminary experimental results have suggested that there may be amino terminal heterogeneity of the Portuguese amyloid protein as well as internal similarity of CNBr derived peptides.

A Beckman microsensitivity amino acid analyzer was procured and installed for the purpose of carrying out primary structure determinations at the carboxyl portions of human amyloid and nonamyloid SAAL and murine amyloid susceptible and amyloid resistant SAAL by the subtractive Edman method. (Drs. Sipe and Sobiech)

The following studies have been conducted in the Section on Cytogenetics (Dr. Tjio and staff) during the past year.

Cytogenetics of Hemtopoetic Disorders: In collaboration with Dr. Germain (University Claude-Bernard, Lyon, France), cytogenetic studies have been carried out using the sister chromatid exchange (S.C.E.) frequencies as a criterion to compare chromosomal alterations found in (1) constitutional hemopathies associated with leukemia or preleukemia, and (2) abnormalities of the hematopoetic system induced by leukemogenic substances such as benzene and related polycyclic hydrocarbons with normal, healthy subjects as controls.

The results of these studies are being worked out. (Dr. Tjio)

Experiments on Pluripotential and Committed Hemopoietic Stem Cells: As a joint point project with Dr. G. Brecher of the Department of Laboratory Medicine, University of California at San Francisco, experiments are being conducted to test a new hypothesis concerning the kinetics of pluripotential stem cells (PSC). It is based on the assumption that the pluripotential stem cells are normally not in cycle. The implications of this thesis are that (1) CSC (committed stem cells) compartments are largely self-maintaining and capable of meeting transient demands for increased hemopoeisis, (2) non-cycling PSC explain the failure of normal marrow to take in syngeneic recipients, and (3) "turning on" the PSC will permit seeding normal syngeneic recipients with normal marrow.

In order to verify this hypothesis, a series of experiments have been conducted in which we have been able to show that normal PSC survive in normal recipients, at least for a day, and may proliferate massively. Bone marrow from female C57Bl and C3H mice was infused into isogeneic recipients of the opposite sex, which were exposed to a uniformly lethal dose of X-irradiation one hour later, with the liver and spleen shielded. By using the C-banding technique, the male and female metaphase cells can be identified in the spleen and marrow of the host animals. Experiments are now under way to determine how long after transfusion the normal cells can be induced to proliferate, whether modalities other than X-ray many "turn on" the trans-fused cells, and whether cells turned on in the donor could proliferate on subsequent transfusion into normal hosts. (Dr. Tjio)

Meiotic Chromosomes of the Female Mouse: This study will be continued. Findings, thus far obtained, of the rate of meiotic nondisjunction in female mice heterozygous for three Robertsonian translocation chromosomes are very similar to those observed in the male meiosis. This was also the case in G.P. mice and in our 34 chromosome strain. Experiments will be carried out to determine which factors may cause or play a role in inducing nondisjunction during anaphase I and/or anaphase II (i.e. age, heterozygous conditions, viral infection, X-ray, and chemicals). (Dr. Tjio)

Fluorescent in Situ Hybridization for Gene Mapping: This study is a col-laborative project with Dr. P. Tischler, Harvard Medical School, Boston and Dr. B. Forget, Yale Medical School, New Haven. Experiments will be carried out to validate a new method for mapping genes on chromosomes. This method, developed in Dr. Tischler's laboratory, involves the *in situ* hybridization of the poly-adenylic acid tail of the RNA to a fluorescent sphere containing poly-uridelic acid. The technique will be used to study the mouse globin genes since these have been mapped by conventional techniques. Metaphase chromosome preparations are initially stained with quinacrine mustard (Q.M.), and each chromosome identified. These same chromosomes are then hybridized to the messenger RNA and the fluorescent spheres are noted. We anticipate that the spheres will detect $\alpha$-globin messenger RNA bound to chromosome 7 and $\beta$-globin messenger RNA bound to chromosome 11. (Dr. Tjio)

Clinical Cytogenetic Studies of Human Congenital and Developmental Disorders: Patients referred to our laboratory for karyotypic diagnosis are those with

various unusual genetic disorders, abnormalities in gonadal development or function, and selected cases of infertility in the male. In these types of cases, we are looking for minor variants or abnormalities as well as gross morphological changes. With routine karyotyping many of these anomalies could be overlooked or misinterpreted; therefore, all preparations from such patient referrals are Giemsa- and quinacrine-banded. Additional procedures such as centromeric-banding, sister chromatid exchange, and/or late replicatio studies are used if a chromosome abnormality is found or suspected. With the addition of new and improvement of our older, more standard banding procedures, we are now better prepared to detect karyotypic variations and more capable of correctly interpreting them. (Dr. White)

Murine Immunogenetic and Cytogenetic Studies: Experiments are currently being performed in collaboration with Dr. A. Steinberg (NIAMDD) to determine the effects of the NZB genetic background on the immunological and cytogenetic status of the NZB/DBZ $F_1$ hybrid and backcross mice. The NZB strain is the mouse model for the human autoimmune disease, lupus erythematosus. Direct chromosome preparations of NZB female spleens have shown that all adult females tested have aneuploidy; this was not the case in direct chromosome preparations of the bone marrow. In contrast, NZB male spleen direct chromosome preprations showed little, if any, aneuploidy. $F_1$ hybrid mice of both sexes also showed a normal chromosome complement in both the bone marrow and the spleen. Backcross animals are currently being investigated for the presence of aneuploidy. Thus far, only backcross females produced by crossing $F_1$ hybrids with NZB mice have shown any aneuploidy. Further investigations are under way to determine if this chromosomal instability is a genetically inherited trait and what role hormones play in influencing this instability.

These same mice have also been studied for immunological abnormalities. The parent NZB strain produces autoantibodies which are cytotoxic for thymocytes. NZB/DBZ $F_1$ females in 50% of the cases had a positive titer of naturally occurring thymocytotoxic antibodies, while intact males had a 0% incidence. Castration of the males increased the incidence of thymocytotoxic antibodies to the level of their female littermates, but removal of the ovaries from female $F_1$ hybrids did not alter the response. Backcross animals showed a similar sex distribution. Backcrosses to the NZB parent had a higher incidence of thymocytotoxic autoantibodies than did the $F_1$ hybrids, but a lower incidence than the NZB parent. Backcrosses to the DBA parent had a lower incidence of thymocytotoxic autoantibodies than did the $F_1$ hybrids, but a higher incidence than the DBA parent.

Both the immunologic and cytogenetic abnormalities seen in NZB mice and their hybrids seems to be under genetic and hormonal control. (Dr. White)

The studies in the Section on Biophysical Histology (Dr. Feder and staff) on histochemical: principles, methods, and applications.

Marking Neurons for Light Microscopy: Studies with two highly fluorescent tissue-reactive dyes (N-substituted naphthalimides) are in progress. The dyes, which are now synthesized on a large scale and in high yield, are analytically pure. They have been given the names Lucifer Yellow CH and VS. (a) These dyes have been used to mark individual neurons in vivo through

recording microelectrodes. Successful marking of a wide variety of vertebrate and invertebrate neurons has now been carried out in laboratories at the N.I.H. and elsewhere. (b) The occurrence of clusters of coupled neurons has been confirmed by injection of a single neuron and observing spread of the dye to contiguous neurons. (c) By injection of dye into the eye of the chick embryo, it has been possible to demonstrate reverse axonal transport along the optic nerve to the isthmo-optic nucleus. Two further studies are in progress. (d) The naphthalimide dyes are under investigation as labels of $\gamma$-globulin and other proteins. The high fluorescence efficiency of the dye and certain other properties make it promising as a label for proteins. (Mr. Stewart)

Inheritance of Nerve Cell Shape: Lucifer Yellow CH is being used to examine neurons in inbred and wild populations of the planorbid snail Biomphalaria glabrata in an attempt to study the inheritance of nerve cells shape. (Dr. Feder and Mr. Stewart)

The following studies have been conducted in the Section on Cellular Function and Ultrastructure (Dr. Lois W. Tice and staff) during the past year.

Immunocytochemistry of COMT: The immunocytochemical distribution of catechol-o-methyl transferase (COMT) has been examined in representative rat tissues using an antibody raised to soluble COMT from rat liver and an indirect labelling technique. Although COMT is abundant in rat brain and other nervous tissues, where it is believed to function in local inactivation of catecholamines released as transmitters from terminals of catecholamine-containing neurons, localization in these tissues was not obtained with this antibody. Instead, antigenic sites were found diffusely throughout the cytoplasm of hepatocytes, proximal and distal convoluted tubules of the kidney, where COMT might be expected to function in o-methylation of circulating catechols. In addition, COMT was present in several sites in the reproductive tract, including epithelia of both oviduct and ductus deferens. This localization lends further credence to the idea, already advanced, that COMT may have an important function in inactivation of catecholestrogens. Further investigations will be required to determine whether the failure to localize COMT in brain is due to different antigenicities of COMT from this source. (Dr. Tice)

Biomechanic and other related studies: Flow visualization with stereo shadowgraphs of stratified fluid will be presented to the International Symposium on Flow Visualization at Tokyo, Japan 11-15 October 1977.

Dr. McCutchen is presently doing an experiment to measure, very indirectly, the increase that swimming motions cause in the skin drag of a fish. He uses the rate of cooling of a hot copper fish model as the analog of skin drag on a real fish, and a permanently bent model as the analog of a fish that swims. If the fish were very slender, the fluid would receive the same sort of push at the peaks of the propulsive wave that travels aft on his body as it does around the bent fish model. Preliminary results suggest that the drag increment is about 30% rather than about 300% as suggested by M. J. Lighthill in 1971 (Large amplitude elongated body theory of fish locomotion.

Proc. Royal Soc. Lond. B 179, 125-128).

Last years report mentioned the fact that coone partially anticipated Weis Fogh's discovery of the clap-fling mechanism, by which some insects may avoid forming starting vortices by commencing their wingbeats with the wings touching. This remains unpublished because Weis Fogh committed suicide before he could present his side of the story.

Dr. McCutchen has shown that the winged seeds, or samaras, of ash and tulip trees do not descend with the simple autogyro autorotation of maple keys, but superpose on it the angle of attack autorotation of a dropped playing card -- such as it is used by ailanthus seeds. With model experiments he has compared the seed dispersing effectiveness of these three strategems ash, maple, and ailanthys, and a fourth, the Zanonia, which forms its seeds as large light gliders.

The joint lubrication chapter for "The Joints and Synovial Fluid" (Leon Sokoloff, Ed., Academic Press) is now finished. In it Dr. McCutchen reported the results of a simple experiment in which the friction of water-lubricated bearings of glass rubbing against wrung-out cartilage and neoprene was observed as a hydrostatic pressure of 100 atmospheres was superposed on the bearing. No change in friction could be detected, in spite of the fact that the pressure across the rubbing surfaces had been increased enormously. This apparently suprizing result accords with the adhesion theory of friction, which says that bearing pressure causes friction only by bringing the rubbing surfaces into real contact, and that it is the force needed to shear the resulting fused junctions that constitute friction.

In "How good intentions made a moster: The grant system and what to do about it, "Dr. McCutchen reviewed the history and consequences of large scale government support of science. The article maintains that the present system of project grants has been evolved to look businesslike and fair, but that as real science is gambling rather than business the effect has been to force grant-seeking scientists to abandon science and, instead, do predicatble exercises that display the laws of nature. (Dr. McCutchen)

Molecular Requirements for Boundary Lubrication: Chemical synthesis of a number of different molecular analogs of the putative biological boundary lubricant, hyaluronate-protein, was tried using macromolecular polycarboxylic acids and globulin proteins coupled with carbodiimides. None lubricated in the friction testing apparatus. This negative result has many possible interpretations that are unresolved. Had the attempt succeeded, the synthetic system would have allowed selective manipulation of components in order to clarify the molecular requirements for boundary lubrication. (Dr. Wilkins)

Boundary Lubricating Films: An expected feature of natural boundary lubricating films adsorbed from synovial fluid onto articular cartilage has been considered. The inherent tendency not to adhere to the apposed and identical film only about 10 nm removed, helps the hyaluronate deposit resist the large shearing forces developed in joint movement, thereby preserving its integrity. (Dr. Wilkins)

Molecular Mechanism for Boundary Lubication: A controversial view of the molecular mechanism involved in boundary lubricating the sliding joints of the skeleton is being studied. The essential elements of this theory are:

1. Anchoring of hyaluronate-protein to the adsorbing surface through tight adhesion of the protein component. Without this firm attachment, the cartilage surface can be stripped of lubricant by shearing forces generated during joint movement.

2. The adsorption of hyaluronate-protein, with its many carboxylate groups, increases the area concentration of negative electrical charges on the cartilage surface. Two such charged surfaces mutually repel, and when prssed together by forces typical of joint loading, they cannot approach one another closer than about 100 Å. This gap, filled with physiological electrolyte, can provide a viscous drag "friction" in the 0.01-0.02 range commonly observed for boundary lubrication by synovial fluid, as long as at least one of the rubbing surfaces is soft, and able to ride over the asperities in the mating surface in order to maintain the thin 100 Å fluid layer. The associated electrostatic floatation calculations need more work. Surface potentials of 50-80 millivolts are expected for articular cartilage covered with adsorbed hyaluronate-protein. Such values are biologically reasonable. Mathematical treatment is complicated by the inapplicability of the usual Debye-Huckel theory to surface potentials greater than 25 mv.

3. Adsorbed hyaluronate-protein has an additional property, that of nonseisure with similar surfaces, which is a seemingly general characteristic of dilute hydrogels. This effect discourages cross-joint entanglement and locking of the absorbed lubricant film pair. Tentative local microseizures across the sliding joint should separate at the coupled gel's outer loops with very little film damage, as a consequence of weakest link breakage. (Dr. Wilkins)

Synthetic Synovial Fluid: The 4th area of research concerns adsorbed, high molecular weight polyacrylic acid's successfully imitating synovial fluid's boundary lubrication of rubber sliding on glass. Polyacrylic acid is one of the few compounds to do this. In contrast to hyaluronate-protein, it is a relatigely simple molecule, without biochemical contaminants. Physical chemists have studied it extensively as a model for polyelectrolyte behavior. (Dr. Wilkins)

The studies in Section on Chemical Pathology (Dr. Friedman and staff) have been concerned with various aspects of cell-virus interactions.

Function of DNA virus genomes in animal cells: Adeno-associated viruses (AAV) are defective parvoviruses. Because of the small size of their DNA genomes and unusual mode of segregation of complementary DNA strands to separate virions, the AAV offer a unique situation amenable to biochemical analysis. AAV synthesizes only a single mRNA species (for its coat protein). Thus the AAV genome seems to consist of a single unique gene enclosed by DNA regions containing reiterated sequences. We are studying AAV as a model system for eukaryotic gene expression. The main objectives of this study are: to analyse the structure of AAV DNA and obtain an accurate transcription map; to study the control of synthesis, post-transcriptional processing and modification and transport and degradation of AAV; to study interactions between AAV and its helper adenovirus in both permissive and non-permissive

cells. In order to analyse the interaction between AAV and adenovirus we require additional information directly relating to adenovirus function in infected cells. We have therefore initiated studies on various parameters of the adenovirus growth cycle. (Drs. Carter, Cook, de la Maza, Handa, and Laughlin).

Major findings since the last report are:

1. Structural Analysis of AAV DNA: Duplex AAV-2 DNA was digested with SalI, PstI, or HaeII restriction endonucleases and the cleavage sites were mapped. SalI cleaves AAV DNA at 0.310 map units, PstI at 0.106, 0.422, and 0.914 and the five HaeII sites were mapped at 0.110, 0.156, 0.181, 0.536, and 0.600 map units. These cleavage products will be useful for the isolation of specific regions from the AAV DNA, located outside of the stably transcribed region of the genome, and also helped to map more complex restriction enzyme cleavages.

Linear duplex molecules of adeno-associated virus type 2 (AAV2) DNA were cleaved with the restriction endonucleases R.HhaI, R.HaeIII, R.HpaII, and R.AluI each of which produced about 20 fragments. These fragments were analysed by electrophoresis in polyacrylamide gel slabs and their physical order on the AAV genome map was determined. This work provides a map of approximately 75 additional cleavage sites on AAV DNA. The inverted terminal repetitious sequence was estimated to be 80 to 120 nucleotides. The results obtained, considered together with previous data, are inconsistent with the model of two permutations of AAV DNA strands having different startpoints. Rather, it appears that the "permutation" in AAV DNA results from an unusual secondary structure located within the inverted repetition, between 20 and 100 nucleotides from the terminus.

The total restriction map of AAV now provides more than 100 cleavage sites which have been accurately mapped. This allows very precise mapping of potential control regions such as transcription promoters or terminators. It has also allowed characterization of incomplete or variant molecules of AAV (see below). (Dr. de la Maza and Carter)

2. Structure of the DNA from Incomplete AAV Particles: Adeno-associated virus-2 (AAV-2) particles containing incomplete genomes were separated from those containing complete viral genomes in cesium chloride density gradients and/or sucrose velocity sedimentation. The incomplete particles banded in the CsCl isopycnic centrifugation at densities of approximately 1.40 and 1.36-1.33 g/cc. Incomplete particles with a density of 1.40 g/cc yielded two main types of DNA (types I and II) having molecular weights of $1.4 \times 10^6$ and $0.8 \times 10^6$ respectively in both neutral and alkaline pH. As assayed with S1 nuclease, at least 70% of the type I and II molecules are cross-linked and probably represent an inverted repetition of a restricted portion of the AAV genome. Restriction endonuclease cleavage with EcoR1, Alu, HhaI, HpaII, HaeIII, and HaeII showed that type I molecules contain 35-50% and type II contain 20-30% of either the left-hand end or right-hand end of the AAV genome. Both "permutations" of the terminal restriction fragments obtained from complete AAV DNA were also present in the incomplete molecules. Incomplete DNA molecules from particles banding at a density of 1.36-1.33 g/cc (type III) had single strand molecular weights of $0.2\text{-}0.6 \times 10^6$. These

molecules contained up to 20% of the genome sequences from one or both termini and both terminal "permutations" were present. Type III molecules appear to be a mixture of both internally deleted molecules and hairpin structures analogous to type I and II. The smallest incomplete molecules contained only 3-5% of the terminal region of the complete genome. The heterogeneous size of incomplete molecules is due to variations in the amount of the internal region of the AAV genome present. Thus restriction enzyme cleavage results in a gradient of fragment concentrations such that terminal fragments are represented in highest yield and those furthest from the termini in lowest molar yield. Since every molecule appears to contain an AAV genome terminus which is believed to be the site of the origin of DNA replication, then at least the snap-back incomplete molecules may arise from aberrant DNA replication rather than by a recombinational mechanism.

These incomplete molecules are now being used to analyse the mechanism of AAV replication as well as adenovirus replication. More importantly, these incomplete molecules have turned out to be very useful probes for analysing the mechanism of the inhibition by AAV of oncogenesis mediated by adenovirus type 12 in newborn hamsters. (Drs. de la Maza and Carter)

3. AAV RNA Transcription: We have continued our studies on transcription of AAV mRNA *in vivo* using human KB cells infected with AAV and Ad2. We have also initiated *in vitro* studies on AAV transcription in an attempt to reconstruct the cellular transcription machinery.

Previous studies, which showed that stable AAV RNA synthesized *in vivo* anneals only to the DNA minus strand between 0.18 and 0.88 map units, may not have detected unstable or nascent RNA. Therefore, AAV-infected KB cell lysates were fractionated by high salt precipitation into a pellet containing nascent (chromatin-associated) RNA and a supernatant containing non-chromatin associated RNA. AAV RNA sequences in each fraction were hybridized with separated strands or restriction fragments of viral DNA. Supernatant RNA contained the same sequences as the previously mapped stable RNA. Chromatin-associated RNA contained the same sequences as supernatant RNA as well as sequences mapping to the left (upstream) of 0.18 and to the right (downstream) of 0.88 map units. Neither RNA fractions contained sequences complementary to the plus DNA strand. Thus, AAV RNA transcripts may be cleaved at the 5' end prior to methyl capping and at the 3' end prior to polyadenylation.

We have begun analysis of AAV RNA synthesis in an *in vitro* system derived by sarkosyl lysis of infected cell nuclei in 0.1 M salt. This released at least 50% of the AAV viral transcription complex (VTC) but little, if any, cellular or adenovirus transcription activity. Solubilized complexes required all 4 nucleoside triphosphates and $Mn^{++}$, were maximally active at 23°C, and were completely inhibited by actinomycin D or α-amanitin at 2 μg/ml. Thus AAV RNA is synthesized by RNA polymerase II. Since the VTC activity was insensitive to heparin, RNA chain initiation did not occur. The VTC sedimented in sucrose gradients at about 16-20S on the leading edge of the AAV replicating molecules. A second VTC peak at 10S may be complexes in which incomplete AAV molecules are the template.

AAV DNA was transcribed in vitro with purified KB cell polymerase II. Denatured DNA was twice as efficient as native DNA as a template. RNA from both templates was about 30% resistant to ribonuclease digestion. Also, separated plus or minus strands of AAV DNA were equally good templates. These findings indicate that transcription was symmetric. The RNA synthesized was heterogenous in size but some nearly full length transcripts were present. (Drs. Carter, Laughlin, Handa and Cook).

Studies on interferon inhibition of murine RNA tumor viruses: Scanning electron microscopy of AKR cells chronically infected with AKR mouse leukemia virus revealed that the number of budding virions was greatly increased in interferon-treated cells. These results, together with previous biochemical findings, suggest that in this system, interferon inhibits a late stage of virus assembly or release.

Murine leukemia virus (MuLV) $ts_3$ is a temperature-sensitive mutant of Moloney MuLV which harbors a defect in a late stage of virus assembly. Cells chronically infected with $ts_3$ accumulate budding virus particles on the plasma membrane when incubated at the nonpermissive temperature. Since interferon has been shown to inhibit the production of oncornaviruses and a similar late stage of virus assembly has been suggested as the site of interferon action, the temporal relationship between the two inhibitions was examined. It was found that the process of virus assembly affected by the inhibitory action of interferon preceded that affected by the temperature-sensitive defect. Analysis of virus yields by multiple parameters indicated that interferon treatment at the nonpermissive temperature resulted in the release of noninfectious viral particles.

The mode of action of interferon in de novo infection of Moloney murine leukemia virus (Mo-MuLV) infection of mouse bone marrow/thymus (TB) cells was also studied. Results indicate that in interferon-treated cells, there is approximately a 2000-fold decrease in the production of infectious MuLV, but only a 10-20 fold decrease in the level of viral specific extracellular reverse transcriptase activity, and only about a 2-fold difference in the number of virus particles observed on the cell membrane as determined by scanning electron microscopic (SEM) studies. Transmission electron microscopic (TEM) studies showed that the proportion of early budding virions, which have shallow crescent-shaped ribonucleoprotein cores to virions in later stages of assembly is relatively higher in interferon-treated cells than in the untreated controls. From a temperature shift-down experiment on a temperature-sensitive mutant of MuLV, ts 3, which produces viral particles that fail to dissociate from the cell surface at the nonpermissive temperature it was demonstrated that ts 3 virions partially assembled on the cell membrane prior to the addition of interferon are able to complete assembly and to dissociate from the cell membrane on temperature shift-down in the presence of interferon action. The data suggest that interferon neither inhibits the late stages of virion assembly at which ts 3 virions are arrested at the nonpermissive temperature, nor prevents release of the virions. In interferon-treated cells, most of the extracellular virions are noninfectious.

Moloney murine leukemia virus produced in interferon-treated mouse thymus and bone marrow cells has a high particle to infectivity ratio. This virus contains a prominent glycoprotein with a molecular wight of about 85,000. This large glycoprotein is only a very minor component of Moloney leukemia virus produced in control TB cells and may be an uncleaved precursor to gp 69-71.

The effect of interferon on glucocorticoid-stimulated mouse mammary tumor virus expression in a murine mammary tumor cell line, Mn5mt/$C_1$, was investigated. Interferon at a concentration of 10-100 IU/ml causes a 5-10 fold inhibition of virus production. The effect has the typical time course of interferon action, is dose dependent, reversible, and not associated with depression of cellular synthesis of macromolecules. The mechanism appears complex. In contrast to the interferon-mediated inhibition of murine leukemia virus, cellular RNA-dependent DNA polymerase (RDDP) is significantly reduced. The ratio of cellular to extracellular RDDP is, however, increased and expression of mouse mammary tumor virus antigens on the cell membrane is not inhibited. The evidence therefore suggests effects of interferon on translation of some viral proteins as well as on viral assembly and release. (Drs. Friedman, Chang, Wong, Myers, Young and Strauchen)

Studies on changes in the plasma membrane of interferon-treated cells: $^{125}$I-labeled TSH and $^{125}$I-labeled cholera toxin could bind to preparations of mouse L-cell plasma membranes. The binding was specific in that it was prevented by unlabeled thyrotropin or cholera toxin, but not by insulin, glucagon, prolactin, growth hormone, human chorionic gonadotropin, or luteinizing hormone.

Mouse interferon inhibited $^{125}$I-labeled TSH binding to L-cell plasma membranes. The effect of mouse interferon on $^{125}$I-labeled cholera toxin binding was more complex, inhibition occurring only after an initial enhancement at low interferon concentrations. A 10-fold higher concentration of interferon was required to inhibit $^{125}$I-labeled cholera toxin binding as compared to $^{125}$I-labeled TSH binding. Mouse interferon was also able to displace bound $^{125}$I-labeled TSH, but not bound $^{125}$I-labeled cholera toxin. The interferon interaction with cell membranes was temperature-sensitive.

Human interferon could induce changes in binding of $^{125}$I-labeled cholera toxin to mouse L-cell plasma membranes similar to those induced by mouse interferon. Mouse interferon induced similar changes in plasma membranes of human KB-3 cells, which are insensitive to both human and mouse interferons.

Interferon treatment also appears to induce a number of changes in the plasma membrane of uninfected AKR,$C^-$ (AKR-2B) mouse cells. Not only was there an apparent increase in the number of intramembranous particles in response to treatment with interferon but also the kinetics of the increase followed that of the establishment of the antiviral activity. Furthermore, both the intramembranous particle density and the antiviral activity were found to decrease to control level by forty-eight hours after the removal of interferon. The bouyant density of plasma membrane was also found to be significantly increased in interferon-treated cells. (Drs. Friedman, Chang, Kohn, Grollman and Lee)

Interferon and chronic viral diseases: $L_y$ cells were treated with 100 reference units per ml of mouse interferon and then infected with a wild-type vesicular stomatitis virus (VSV) at a multiplicity of 10-60 PFU per cell. Prolonged infection of cultures, ensued, lasting from 14 to at least 60 days. Less than 1% of the cells produced infectious virus, but more than 10% produced detectable levels of VSV antigens. No small virion RNA forms (< 42S) characteristic of defective interfering virus particles were detected. The virus produced appeared to be temperature sensitive. There was decreased plaquing efficiency at 37°C or 39°C compared to 32°C and termination of the chronic infection due to CPE within a few days after shift to 32°C. The cultures resisted superinfection with wild-type VSV or with a heterologous virus, encephalomyocarditis (EMC) virus. Treatment of cultures with rabbit anti-mouse interferon globulin resulted in a marked increase in virus titers and termination of the chronic infection. Prolonged VSV infection in this system may be related both to the emergence of temperature-sensitive mutants and to endogenous interferon production rather than to cyclical generation of DI particles. (Dr. Friedman and Ms. Ramseur)

SV40-specific ribosome-binding proteins induced by a nondefective adenovirus 2-SV40 hybrid: We have studied the intracellular distribution of the two SV40-specific proteins, with apparent molecular weights of 56,000 and 42,000, detectable in human KB cells infected by a nondefective adenovirus 2-simian virus 40 hybrid, $Ad2^{+}ND_2$. After a twenty-minute pulse of [$^{35}$S]-methionine, about two-thirds of the newly-synthesized 56K protein and one-third of the 42K protein were found to be localized on the plasma membrane. The remainder of each protein was found in the cytoplasm, while the nuclear fraction was virtually free of either component. A significant portion of both proteins present in the cytoplasmic fraction was complexed to the 40S ribosomal subunits, and was not removed by treatment with 0.5M KCl. Moreover, the portion that was found free in the cytoplasm could bind preferentially and quantitatively to purified 40S ribosomes in vitro, leading us to propose that these SV40 proteins may act as translational control elements in the cell. (Drs. Jay and Jay)

| SMITHSONIAN FOREIGN EXCHANGE PROJECT NUMBER (DO NOT use this space) | U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT | PROJECT NUMBER ZO1 AM 21,001-03 LEP |
|---|---|---|

PERIOD COVERED

July 1, 1976 through September 30, 1977

TITLE OF PROJECT (50 characters or less)

Preparation of stained tissue sections for investigation and diagnostic purposes.

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

Virginia Reveal — Head, Tissue Preparation Lab

COOPERATING UNITS (if any)

None

LAB/BRANCH

Laboratory of Experimental Pathology

SECTION

Tissue Preparation Section

INSTITUTE AND LOCATION

NIAMDD, LEP, Bethesda, Maryland

| TOTAL MANYEARS: | PROFESSIONAL: | OTHER: |
|---|---|---|
| 1.5 | 0 | 1.5 |

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The tissue laboratory will continue to provide technical assistance to the investigators of the Laboratory of Experimental Pathology and to those of other units at the NIH who require technical help. A considerable part of the tissue laboratory's work is concerned with providing a wide variety of special histologic techniques adjusted to the various research projects as shown below.

| | Specimen Accessioned | Stained Slides Routine | Stained Slides Special | Spare Slides | Total |
|---|---|---|---|---|---|
| Animals | 960 | 1612 | | | |
| Surgicals | 6 | 6 | | | |
| | | 1618 | 1502 | | |
| | | | 3120 | 3877 | |
| | | | | | 6997 |



P 1-0940
(Rev. 12-76)

| SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space) | U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT | PROJECT NUMBER ZOI AM 21,002-4 LEP |
|---|---|---|

PERIOD COVERED
July 1, 1976 through September 30, 1977

TITLE OF PROJECT (80 characters or less)

Immunohistochemistry of COMT

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

| | | |
|---|---|---|
| PI: | Lois W. Tice | Chief, Section on Cellular Function and Ultrastructure |
| OTHER: | Katsuhiro Inoue | Visiting Fellow |

COOPERATING UNITS (if any)

Dr. C. R. Creveling, LC:NIAMDD

LAB/BRANCH
Laboratory of Experimental Pathology

SECTION
Section on Cellular Function and Ultrastructure

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland

| TOTAL MANYEARS: | PROFESSIONAL: | OTHER: |
|---|---|---|
| 3 | 2 | 1 |

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The distribution of catechol-o-methyl transferase in various rat tissues was studied using immunocytochemical methods.

Project description:

Immunocytochemistry of COMT: The immunocytochemical distribution of catechol-o-methyl transferase (COMT) has been examined in representative rat tissues using an antibody raised to soluble COMT from rat liver and an indirect labelling technique. Although COMT is abundant in rat brain and other nervous tissues, where it is believed to function in local inactivation of catecholamines released as transmitters from terminals of catecholamine-containing neurons, localization in these tissues was not obtained with this antibody. Instead, antigenic sites were found diffusely throughout the cytoplasm of hepatocytes, proximal and distal convoluted tubules of the kidney, where COMT might be expected to function in o-methylation of circulating catechols. In addition, COMT was present in several sites in the reproductive tract, including epithelia of both oviduct and ductus deferens. This localization lends further credence to the idea, already advanced, that COMT may have an important function in inactivation of catecholestrogens. Further investigations will be required to determine whether the failure to localize COMT in brain is due to different antigenicities of COMT from this source.

| SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space) | U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT** | PROJECT NUMBER ZOI AM 21,003-3 LEP |
|---|---|---|

PERIOD COVERED
July 1, 1976 through September 30, 1977

TITLE OF PROJECT (80 characters or less)
Biomechanics and related studies

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

C. W. McCutchen Research Physicist

COOPERATING UNITS (if any)

none

LAB/BRANCH
Laboratory of Experimental Pathology

SECTION
Section on Cellular Function and Ultrastructure

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20014

| TOTAL MANYEARS: | PROFESSIONAL: | OTHER: |
|---|---|---|
| 1/2 | 1/2 | 0 |

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Studies have been pursued concerning the swimming of fish, and other examples of the fluid dynamics of living things.

A simple but interesting experiment has shown that raising the hydrostatic pressure in water bathing bearings of glass against wrung-out cartilage or neoprene has no noticable effect on the friction.

Prolonged thinking has convinced McCutchen that most of the troubles with the grant system result from the understandable but unwise attempt to treat science as a business.



PHS-6040

Project description:

Flow visualization: "Flow visualization with stereo shadowgraphs of stratified fluid" will be presented to the International Symposium on Flow Visualization at Tokyo, Japan 11-15 October 1977.

Drag increment of a swimming fish: Experimently are in progress to measure, indirectly, the increase that swimming motions cause in the skin drag of a fish. The rate of cooling of a hot copper fish model is used as an analog of skin drag on a real fish, and a permanently bent model as the analog of a fish that swims. If the fish were very slender, the fluid would receive the same sort of push at the peaks of the propulsive wave that travels aft on his body as it does around the bent fish model. Preliminary results suggest that the drag increment is about 30%, rather than about 300% as suggested by M. J. Lighthill in 1971 (Large amplitude elongated body theory of fish locomotion. Proc. Royal Soc. Lond. B 179, 125-128).

Clap-fling mechanism: The fact that Cone partially anticipated Weis' Fogh's discovery of the clap-fling mechanism, by which some insects may avoid forming starting vortices by commencing their wingbeats with the wings touching, remains unpublished. Weis Fogh committed suicide before he could present his side of the story.

Winged seeds: I have shown that the winged seeds, or samaras, of ash and tulip trees do not descend with the simple autogyro autorotation of maple keys, but superpose on it the angle of attack autorotation of a dropped playing card -- such as is used by ailanthus seeds. Using model experiments I have compared the seed-dispersing effectiveness of the three strategems, ash, maple, and ailanthus, and a fourth, that of the Zanonia, which forms its seeds as large, light gliders.

Hydrostatic pressure does not affect bearing friction: The joint lubrication chapter for "The Joints and Synovial Fluid" (Leon Sokoloff, Ed., Academic Press) is now finished. In it I report the results of a simple experiment in which the friction of water-lubricated bearings of glass rubbing against wrung-out cartilage and neoprene was observed as a hydrostatic pressure of 100 atmospheres was applied to the bearing. No change in friction could be detected, in spite of the fact that the pressure pushing the rubbing surfaces together had been increased enormously. This apparently suprising result accords with the adhesion theory of friction, which says that bearing pressure causes friction only by bringing the rubbing surfaces into real contact, and that it is the force needed to shear the resulting fused junctions that constitutes friction.

Support of science: In "How good intentions made a monster: The grant system and what to do about it," I review the history and consequences of large scale government support of science. I maintain that the present system of project grants has been evolved to look businesslike and fair, but that as real science is gambling rather than business, the effect has been to force grant-seeking scientists to abandon science and, instead, to do predictable exercises that display the laws of nature.

Publications:

1. McCutchen, C. W. Comment on The fundamental fluid transport mechanisms through normal and pathological articular cartilage during function. I. The formulation and II. The analysis, solution and conclusions, by P. A. Torzilli and V. C. Mow. J. Biomechan. 10: 279-280, 1977.

2. McCutchen, C. W. The spinning rotation of ash and tulip tree samaras. Science (in press).

3. McCutchen, C. W. How good intentions made a monster: The grant system and what to do about it. submitted to The Sciences.

4. McCutchen, C. W. Joint Lubrication. In Human Joints and Synovial Fluid, Leon Sokoloff, Ed., Academic Press, New York (in press).

| SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space) | U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT** | PROJECT NUMBER ZOI AM 21,004-10 LEP |
|---|---|---|

PERIOD COVERED

July 1, 1976 through September 30, 1977

TITLE OF PROJECT (80 characters or less)

Control of viral genetic function in interferon-treated and virus-infected animal cells

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

| | |
|---|---|
| Dr. Robert Friedman | Chief, LEP,<br>Chief, Section on Chemical Pathology |
| Dr. Esther Chang | Visiting Associate |
| Dr. Maureen Myers | Staff Fellow |
| Dr. Francis Jay | Visiting Fellow |
| Ms. Janet Ramseur | Research Biologist |

COOPERATING UNITS (if any)

Dr. Leonard Kohn, Dr. George Lee, Dr. Evelyn Grollman, LPP:NIAMDD, Dr. Timothy Triche, N. Young, S. Strauchen, S. Mims, LP:NCI; G. Jay, A. Levine, POB:NCI; P. Wong, P. H. Yuen, R. MacLeod, University of Illinois.

LAB/BRANCH

Laboratory of Experimental Pathology

SECTION

Section on Chemical Pathology

INSTITUTE AND LOCATION

NIAMDD, Bethesda, Maryland

| TOTAL MANYEARS: | PROFESSIONAL: | OTHER: |
|---|---|---|
| 6 | 4 | 2 |

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

We have employed several systems to investigate the problem of control of biological function at the molecular level in animal cells. For this purpose we have been interested in virus replication and its inhibition since these can be studied at the molecular level. Early functions of an oncogenic virus, SV40, which seem in relation to tumor induction are under study. In the case of interferon, a natural inhibition of virus growth, we have studied murine leukemia virus production in interferon-treated cells. We have also been interested in alterations in plasma membranes induced by interferon treatment in interferon binding sites on the cell surface. Finally, we are examining the role of interferon in the induction and persistence of chronic viral infections.

Project Description:

Studies on interferon inhibition of murine RNA tumor viruses: Scanning electron microscopy of AKR cells chronically infected with AKR mouse leukemia virus revealed that the number of budding virions was greatly increased in interferon-treated cells. These results, together with previous biochemical findings, suggest that in this system, interferon inhibits a late stage of virus assembly or release.

Murine leukemia virus (MuLV) $ts_3$ is a temperature-sensitive mutant of Moloney MuLV which harbors a defect in a late stage of virus assembly. Cells chronically infected with $ts_3$ accumulate budding virus particles on the plasma membrane when incubated at the nonpermissive temperature. Since interferon has been shown to inhibit the production of oncornaviruses and a similar late stage of virus assembly has been suggested as the site of interferon action, the temporal relationship between the two inhibitions was examined. It was found that the process of virus assembly affected by the inhibitory action of interferon preceded that affected by the temperature-sensitive defect. Analysis of virus yields by multiple parameters indicated that interferon treatment at the nonpermissive temperature resulted in the release of noninfectious viral particles.

The mode of action of interferon in de novo infection of Moloney murine leukemia virus (Mo-MuLV) infection of mouse bone marrow/thymus (TB) cells was also studied. Results indicate that in interferon-treated cells, there is approximately a 2000-fold decrease in the production of infectious MuLV, but only a 10-20 fold decrease in the level of viral specific extracellular reverse transcriptase activity, and only about a 2-fold difference in the number of virus particles observed on the cell membrane as determined by scanning electron microscopic (SEM) studies. Transmission electron microscopic (TEM) studies showed that the proportion of early budding virions, which have shallow crescent-shaped ribonucleoprotein cores to virions in later stages of assembly is relatively higher in interferon-treated cells than in the untreated controls. From a temperature shift-down experiment on a temperature-sensitive mutant of MuLV, ts 3, which produces viral particles that fail to dissociate from the cell surface at the nonpermissive temperature it was demonstrated that ts 3 virions partially assembled on the cell membrane prior to the addition of interferon are able to complete assembly and to dissociate from the cell membrane on temperature shift-down in the presence of interferon action. The data suggest that interferon neither inhibits the late stages of virion assembly at which ts 3 virions are arrested at the nonpermissive temperature, nor prevents release of the virions. In interferon-treated cells, most of the extracellular virions are noninfectious.

Moloney murine leukemia virus produced in interferon-treated mouse thymus and bone marrow cells has a high particle to infectivity ratio. This virus contains a prominent glycoprotein with a molecular wight of about 85,000. This large glycoprotein is only a very minor component of Moloney leukemia virus produced in control TB cells and may be an uncleaved precursor to gp 69-71.

The effect of interferon on glucocorticoid-stimulated mouse mammary tumor virus expression in a murine mammary tumor cell line, Mn5mt/$C_1$, was investigated. Interferon at a concentration of 10-100 IU/ml causes a 5-10 fold inhibition of virus production. The effect has the typical time course of interferon action, is dose dependent, reversible, and not associated with depression of cellular synthesis of macromolecules. The mechanism appears complex. In contrast to the interferon-mediated inhibition of murine leukemia virus, cellular RNA-dependent DNA polymerase (RDDP) is significantly reduced. The ratio of cellular to extracellular RDDP is, however, increased and expression of mouse mammary tumor virus antigens on the cell membrane is not inhibited. The evidence therefore suggests effects of interferon on translation of some viral proteins as well as on viral assembly and release.

Studies on changes in the plasma membrane of interferon-treated cells: $^{125}$I-labeled TSH and $^{125}$I-labeled cholera toxin could bind to preparations of mouse L-cell plasma membranes. The binding was specific in that it was prevented by unlabeled thyrotropin or cholera toxin, but not by insulin, glucagon, prolactin, growth hormone, human chorionic gonadotropin, or luteinizing hormone.

Mouse interferon inhibited $^{125}$I-labeled TSH binding to L-cell plasma membranes. The effect of mouse interferon on $^{125}$I-labeled cholera toxin binding was more complex, inhibition occurring only after an initial enhancement at low interferon concentrations. A 10-fold higher concentration of interferon was required to inhibit $^{125}$I-labeled cholera toxin binding as compared to $^{125}$I-labeled TSH binding. Mouse interferon was also able to displace bound $^{125}$I-labeled TSH, but not bound $^{125}$I-labeled cholera toxin. The interferon interaction with cell membranes was temperature-sensitive.

Human interferon could induce changes in binding of $^{125}$I-labeled cholera toxin to mouse L-cell plasma membranes similar to those induced by mouse interferon. Mouse interferon induced similar changes in plasma membranes of human KB-3 cells, which are insensitive to both human and mouse interferons.

Interferon treatment also appears to induce a number of changes in the plasma membrane of uninfected AKR,$C^-$ (AKR-2B) mouse cells. Not only was there an apparent increase in the number of intramembranous particles in response to treatment with interferon but also the kinetics of the increase followed that of the establishment of the antiviral activity. Furthermore, both the intramembranous particle density and the antiviral activity were found to decrease to control level by forty-eight hours after the removal of interferon. The bouyant density of plasma membrane was also found to be significantly increased in interferon-treated cells.

Interferon and chronic viral diseases: $L_y$ cells were treated with 100 reference units per ml of mouse interferon and then infected with a wild-type vesicular stomatitis virus (VSV) at a multiplicity of 10-60 PFU per cell. Prolonged infection of cultures, ensued, lasting from 14 to at least 60 days. Less than 1% of the cells produced infectious virus, but more than 10% produced detectable levels of VSV antigens. No small virion RNA forms (< 42S) characteristic of defective interfering virus particles were detected. The

virus produced appeared to be temperature sensitive. There was decreased plaquing efficiency at 37°C or 39°C compared to 32°C and termination of the chronic infection due to CPE within a few days after shift to 32°C. The cultures resisted superinfection with wild-type VSV or with a heterologous virus, encephalomyocarditis (EMC) virus. Treatment of cultures with rabbit anti-mouse interferon globulin resulted in a marked increase in virus titers and termination of the chronic infection. Prolonged VSV infection in this system may be related both to the emergence of temperature-sensitive mutants and to endogenous interferon production rather than to cyclical generation of DI particles.

SV40-specific ribosome-binding proteins induced by a nondefective adenovirus 2-SV40 hybrid: We have studied the intracellular distribution of the two SV40-specific proteins, with apparent molecular weights of 56,000 and 42,000, detectable in human KB cells infected by a nondefective adenovirus 2-simian virus 40 hybrid, $Ad2^{+}ND_{2}$. After a twenty-minute pulse of [$^{35}$S]-methionine, about two-thirds of the newly-synthesized 56K protein and one-third of the 42K protein were found to be localized on the plasma membrane. The remainder of each protein was found in the cytoplasm, while the nuclear fraction was virtually free of either component. A significant portion of both proteins present in the cytoplasmic fraction was complexed to the 40S ribosomal subunits, and was not removed by treatment with 0.5M KCl. Moreover, the portion that was found free in the cytoplasm could bind preferentially and quantitatively to purified 40S ribosomes in vitro, leading us to propose that these SV40 proteins may act as translational control elements in the cell.

Publications:


1. Kohn, L. D., Friedman, R. M., Holmes, J. M., and Lee, G. Use of thyrotropin and cholera toxin to probe the mechanism by which interferon initiates its antiviral activity. Proc. Natl. Acad. Sci. USA 73: 3695, 1976.

2. Chang, E. H., Mims, S. J., Triche, T. J., Friedman, R. M. Interferon inhibits mouse leukemia virus release: An electron microscope study. J. Gen. Virol. 34, 363, 1977.

3. Chang, E. H., Myers, M. W., Wong, P. K. Y., and Friedman, R. M. The inhibitory effect of interferon on a temperature-sensitive mutant of Moloney murine leukemia virus. Virology 77: 625, 1977.

4. Wong, P. K. Y., Yuen, P. H., MacLeod, R., Chang, E. H., Myers, M. W., and Friedman, R. M. The effect of interferon on de novo infection of Moloney murine leukemia virus. Cell 10: 245, 1977.

5. Chang, E. H., and Friedman, R. M. A large glycoprotein of Moloney leukemia virus derived from interferon-treated cells. Biochem. Biophys. Res. Commun., in press.

6. Strauchen, J. A., Young, N. A., and Friedman, R. M. Interferon-mediated inhibition of mouse mammary tumor virus expression in cultured cells. Virology, in press.

7. Jay, G., Jay, F. T., Friedman, R. M., and Levine, A. S. Simian virus 40-specific ribosome-binding proteins induced by a nondefective Adenovirus 2-simian virus 40 hybrid. J. Virol., in press.

8. Ramseur, J. M., and Friedman, R. M. Prolonged infection of interferon-treated cells by vesicular stomatitis virus. I. Possible role of temperature-sensitive mutants of interferon. J. Gen. Virol., in press.

9. Friedman, R. M., Grollman, E. F., Chang, E. H., Kohn, L. D., Lee, G., and Jay, F. T. Interferon and glycoprotein hormones. Texas Rep. Biol. Med., in press.

| SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space) | U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT | PROJECT NUMBER ZOI AM 21,005-04 LEP |
|---|---|---|

PERIOD COVERED
July 1, 1976 through September 30, 1977

TITLE OF PROJECT (80 characters or less)
Histochemistry: Principles, methods, and applications.

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: N. Feder Chief, Section on Biophysical Histology
Other: W. Stewart Staff Fellow

COOPERATING UNITS (if any)
None

LAB/BRANCH
Laboratory of Experimental Pathology

SECTION
Section on Biophysical Histology

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland

| TOTAL MANYEARS: | PROFESSIONAL: | OTHER: |
|---|---|---|
| 3 | 2 | 1 |

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Cytochemical methods have been used to determine the position of substances in tissues and cells. (1) Two new highly fluorescent tissue-reactive dyes (N-substituted naphthalimides), given the names Lucifer Yellow CH and VS, have been used to mark individual neurons in vivo through recording microelectrodes. (2) Lucifer Yellow CH is being used to examine neurons in inbred and wild populations of the planorbid snail Biomphalaria glabrata in an attempt to study the inheritance of nerve cell shape.



FORM-0040

Marking Neurons for Light Microscopy: Studies with two highly fluorescent tissue-reactive dyes (N-substituted naphthalimides) are in progress. The dyes, which are now synthesized on a large scale and in high yield, are analytically pure. They have been given the names Lucifer Yellow CH and VS. (a) These dyes have been used to mark individual neurons in vivo through recording microelectrodes. Successful marking of a wide variety of vertebrate and invertebrate neurons has now been carried out in laboratories at the N.I.H. and elsewhere. (b) The occurrence of clusters of coupled neurons has been confirmed by injection of a single neuron and observing spread of the dye to contiguous neurons. (c) By injection of dye into the eye of the chick embryo, it has been possible to demonstrate reverse axonal transport along the optic nerve to the isthmo-optic nucleus. Two further studies are in progress. (d) The naphthalimide dyes are under investigation as labels of γ-globulin and other proteins. The high fluorescence efficiency of the dye and certain other properties make it promising as a label for proteins.

Inheritance of Nerve Cell Shape: Lucifer Yellow CH is being used to examine neurons in inbred and wild populations of the planorbid snail Biomphalaria glabrata in an attempt to study the inheritance of nerve cells shape.

Publications.

Feder, N.: Solitary cells and enzyme exchange in tetraparental mice. Nature 263:67-69, 1976.

| SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space) | U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT** | PROJECT NUMBER Z01AM201006-09LEP |
|---|---|---|

PERIOD COVERED

1 July 1976- 30 September 1977

TITLE OF PROJECT (80 characters or less)

Protein and Proteolytic Enzyme Interrelationships in Normal and Disease States.

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

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Dr. D. T. Liu, DBP: BB
Dr. P.P. Costa, Instituto de Neurologia, Porto, Portugal

LAB/BRANCH

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Section on Molecular Pathology

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| TOTAL MANYEARS: | PROFESSIONAL: | OTHER: |
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| 3.5 yrs. | 1 | 2.5 |

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☐ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

It is the purpose of this project to study the chemistry and structure of amyloid and amyloid related proteins. The main topics of present interest are:

1) Isolation and chemical characterization of amyloid fibril protein from various types of systemic amyloidosis and localized amyloid deposits.

2) Mechanism of formation of amyloid deposits by lysosomal enzyme digestion of immunoglobulin precursors.

3) Isolation and purification of proteolytic enzymes involved in immunoglobulin degradation.



PHS-6040

Project Description

## Tissue Localization of An Amyloid Protein

Further studies on amyloid proteins and their origin have utilized a mono-specific anti-AA antibody to demonstrate in tissues by indirect immunofluorescence the site of the AA protein, usually associated with the amyloid fibrils of secondary amyloidosis and reported as a major constituent of the fibrils in a case of familial Mediterranean fever. The antibody stained secondary amyloid deposits as well as intracytoplasmic material in reactive lymphoid tissues. No staining was observed when control antisera were used. AA containing cells were most frequently observed in reactive lymphoid tissues. Tonsils from patients with chronic tonsillitis invariably contained large numbers of positive cells. Spleens showing reactive lymphoid hyperplasia were also frequently positive. Conditions in which the AA positive cells were especially numerous included chronic infections, sprue and Feltys' syndrome. Fewer lymph nodes in which reactive hyperplasia was prominent were studied, but in two such examples, only one was positive. Cells containing AA protein usually had the morphologic characteristics of plasma cells. Their distribution in lymphoid tissue was also like that of plasma cells. In tonsils, they were found in subepithelial and interfollicular zones, in lymph nodes, in the medulla; and in spleens, in the marginal zone and red pulp. Approximately 10-20% of AA-containing cells also contained IgG. These data suggest that the AA protein and immunoglobulin usually are not synthesized or secreted simultaneously. Further studies, perhaps at the electron microscopic level, are needed to demonstrate whether or not AA- containing plasmacytoid cells are in fact plasma cells, and not cells of an independent immunocompetent line. The question of synthesis versus processing or selective membrane reception must also be considered. The question synthesis versus processing or selective membrane reception must also be considered. The appearance of AA-containing cells in the liver was different from those in lymphoid tissues. In liver, the Kupffer cells apparently contained the protein. This could be a consequence of non-specific phagocytosis or the protein could be **processed and/or** secreted by these cells. However, phagocytic cells in lymph nodes and spleens did not appear positive.(E. Jaffe)

## Chemical Analysis of a Familial Amyloid Protein

Techniques **for** the isolation of highly homogeneous amyloid fibrils were taught in this laboratory to a member of the Portuguese research team (Dr. P. Costa) studying the familial Portuguese amyloid neuropathy. A major protein common to all preparations was isolated from these fibrils by Sephadex G-100 gel filtration in 5 M guanidine. This major protein was tested with antibodies to normal human serum which identified it as antigenically related to serum prealbumin. Antibodies **raised** to the amyloid fibril protein were found to react with normal serum prealbumin and appeared to be antigenically identical with it. Differences between this protein (FAP) and prealbumin were noted in its amino acid composition and the molecular weight of its $C^{14}$ carboxymethylated cyanogen bromide fragment (Dr. K. Sobiech). Studies are now in progress to determine the amino acid sequence of the amino terminal cyanogen bromide fragment of FAP.

Publications: ZO1AM201006-09LEP


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2. Linke, R.P., Eanes, E.D., Termine, J.D., Bladen, H.A., and Glenner, G.G.: Formation of amyloid like fibrils from insulin and glucagon in vitro. In Wegelius, O. and Pasternack, A. (Ed): Amyloidosis. New York, Academic Press, 1976, pp. 371-374.

3. Sipe, J.D., McAdam, K.P.W.J., Torain, B.F., and Glenner, G.G.: Conformational flexibility of the serum amyloid precursor SAA. Brit. J. Exp. Path. 57: 582-592, 1976.

4. Glenner, G.G., Biochemic de la substance amyloide. Consequences sur la pathogenie de l'amylose generalisee. Concours Medical, Suppl. 9: 8-11, 1977.

5. Watanabe, S., Jaffe, E., Pollock, S., Sipe, J., and Glenner, G.: Amyloid AA protein: Cellular distribution and appearance. Amer. J. Clin. Path. 67: 540-544, 1977.

6. Ignaczak, T.F., Sipe, J.D., Linke, R.P., and Glenner, G.G.: Immunochemical studies on the nature of SAA and secondary amyloidosis. J. Lab. Clin. Med. 89: 1092-1104, 1977.

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8. Glenner, G.G.: Qu'est-ce que l'amyloidose? Cahiers Medicaux, 2: 2343-2344, 1977.

| SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space) | U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT | PROJECT NUMBER ZOI AM 21,007-04 LEP |
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PERIOD COVERED

July 1, 1976 through September 30, 1977

TITLE OF PROJECT (80 characters or less)

Origin and Structure of an Acute Phase Protein (SAA) Antigenically Related to an Amyloid Fibril Protein (AA).

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: Jean D. Sipe Senior Staff Fellow

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SUMMARY OF WORK (200 words or less - underline keywords)

It is the purpose of this project to study, by biochemical and immunological techniques, the biosynthesis and structural properties of a normal acute phase serum protein, SAA, which has been identified by its cross-reaction with antibodies raised to an amyloid fibril protein, AA. The main topics of present interest are: 1) Structure and conformational properties of SAA; 2) Molecular events of acute phase SAA elevation; 3) Tissue and serum distribution of proteins related to the amyloid fibril protein AA during development of amyloidosis.



2-2040
(Rev. 10-76)

Project Description:

Introduction: Amyloidosis is a group of diseases in which insoluble protein fibrils having a beta pleated sheet conformation accumulate in tissues. Amyloid fibril proteins are thought to be derived from normally innocuous polypeptide precursors by proteolysis or other physiochemical mechanisms. The major fibril protein isolated from human secondary amyloid deposits and from most spontaneous and induced amyloid deposits in experimental animals has been designated AA. Antibodies to denatured AA polypeptides cross-react with a serum protein which has been designated SAA.

Isolation of SAA: The first project, undertaken in 1973, was to purify SAA from human serum by affinity chromatography using anti-AA immunosorbents. These endeavors were unsuccessful because SAA was not retained by the affinity column; this observation was quite puzzling in view of the fact that anti-AA and SAA-containing serum formed strong precipitin lines in Ouchterlony double immunodiffusion. Earlier attempts by Dr. Linke to isolate SAA by conventional size and charge fractionation techniques and by differential precipitation had been unsuccessful because SAA was invariably "lost" after purification, according to Ouchterlony analysis of serum fractions. This investigator recognized the need for a quantitative assay to monitor the isolation of SAA and to study its origin and structure. In collaboration with Dr. Linke an antigenic immunosorbent column was prepared from a highly purified AA preparation of homogenous size and charge and known primary structure at the amino terminus. Antibodies purified from antiserum raised to crude denatured AA fibrils were employed by Dr. Linke to monitor the isolation of SAA (Ref. 1) and by Dr. Watanabe to investigate the cellular distribution and possibly the cellular origin of the AA protein by indirect immunofluorescence (Ref. 9). This investigator, in collaboration with Dr. Ignaczak, developed a solid phase radioimmunoassay for the AA protein which employed purified antibodies to purified AA protein. These antibodies were adsorbed to the wells of flexible microtitration plates and cross-reacting antigens in serum and tissues were determined according to their degree of competition with $^{125}$I-AA. When this quantitative assay was used to monitor the isolation of SAA (Ref. 2) several interesting observations on the structure and properties of SAA were made.

Structure of SAA: The molecular weight of native SAA was determined by the Ouchterlony technique to be 160,000-200,000 and that of guanidine HCl denatured SAA (designated SAAL) to be 12,500 (Ref. 1). When this investigator analyzed by RIA the gel filtration of SAA in guanidine HCl, it was found that 90% of SAA was not dissociated to SAAL, but had remained undetected by double immunodiffusion with anti-AA antibodies (Ref. 2). Formic acid denaturation of SAA-containing serum had been shown by other laboratories to be effective in forming SAAL, and we determined by RIA that 85% of SAA was dissociated to SAAL by this agent.

The above work established that SAA consisted of SAAL complexed either with itself or other polypeptides by noncovalent bonds. One experimental approach to determine whether there was a high molecular weight polypeptide carrier for the SAAL polypeptide, was to radiolabel the SAA fraction from whole serum with iodine 125 and to then mix it with unlabeled, whole, SAA-

containing serum and carry out immunoprecipitation with anti-AA antibodies using a double antibody technique. The immunoprecipitated $^{125}$I-SAA was analyzed by SDS urea-polyacrylamide gel electrophoresis and only a fraction of the precipitated radioactivity was observed to migrate with a molecular weight of 12,500. Multiple radiolabeled species and molecular weights intermediate to SAA and SAAL were also detected and appear to represent incompletely dissociated SAA, suggesting that SAA is an aggregate of several SAAL chains (Ref. 8).

Certain interesting conformational properties of SAA were elucidated by the RIA analysis of SAAL isolation (Ref. 2, 3). It appears that not all determinants which cross-react with antibodies to the AA polypeptide are accessible in native SAA, and that they are exposed during the isolation of SAAL. This phenomenon is not solely due to the formation of a smaller cross-reacting antigen, since formic acid derived SAAL is almost 50 fold more immunoreactive than SAAL prepared by guanidine HCl denaturation. The increase in immunoreactivity of SAA-containing serum was observed to a lesser extent upon storage of serum or experimental manipulation which does not normally dissociate noncovelent bonds.

The sensitivity of RIA for the detection of SAA was lower than expected when AA cross-reactivity values for sera were correlated with their reaction with anti-AA anti-AA antibodies in immunodiffusion. RIA determination of SAA in undenatured whole serum reaches maximum values of the order of 5 μg/ml, yet sera with values as low as 50 ng/ml were strongly reactive in double immunodiffusion. It appears that unfolding of cross-reacting determinants occurs during the process of double immunodiffusion in agarose gel, and this is additional evidence for the "buried" nature of AA determinants in native SAA.

Additional evidence for the conformational flexibility of SAA includes the observation that heterogeneous molecular weight species of SAA are formed during storage of serum at 4°C (Ref. 3). This dissociation and aggregation is accompanied by an increase in immunoreactivity of a constant volume of serum. Because this conformational flexibility may play an important role in the induction of the beta pleated sheet conformation prior to fibril deposition in tissues (if SAA is the circulating precursor of the AA fibril protein) we compared SAA from patients with and without secondary amyloidosis and found that both types exhibit this flexibility in conformation. However, there are differences in susceptibility of the two types of SAA to SAAL formation. SAA from patients with secondary amyloidosis was less readily dissociated to SAAL than was SAA from patients without amyloidosis. Furthermore, isolated SAAL preparations from patients with amyloidosis, although similar in immunoreactivity to that from patients without amyloidosis, have a markedly greater tendency toward reaggregation even in the presence of formic acid. It seems highly significant that SAAL aggregates easily, and is then difficult to dissociate when it has a conformation such that antigenic determinants which cross-react with antibodies raised to the insoluble fibrils deposited in tissues are maximally exposed.

The increase in immunoreactivity of SAA-containing serum which accompanys the formic acid dissociation of SAA to SAAL is time and temperature

dependent (Ref. 3) and is also affected by the concentration of serum in formic acid. Because reproducible inhibition curves can be obtained for a given serum sample after formic acid denaturation, this treatment is routinely employed now for analysis of all serum and tissue samples.

Acute phase nature of SAA: The relative occurence of SAA in serum samples from 500 individuals either healthy or in various disease states, was compared by RIA and double immunodiffusion analysis (Ref. 8). SAA levels were elevated above normal in several categories of neoplastic, inflammatory and infectious diseases as well as in secondary amyloidodis. The lowest median value, 8 ng/nl, was observed for approximately 150 normal sera, with no age related increase in subjects ranging in age from 16 to 70 years. The non-Gaussian distribution of values is consistent with observations in other laboratories that SAA is an acute phase reactant in man; hence the RIA for SAA has no prognostic or diagnostic significance for secondary amyloidosis.

Recent collaborative studies with Drs. McAdam, Elin, and Wolff have shown that acute phase SAA elevations can be produced in previously asymptomatic normal individuals by the nonantigenic pyrogen etiocholanolone, and that the acute SAA response exactly coincides with the acute phase C-reactive protein elevation which has long been used as a clinical marker for inflammmation (Ref. 7).

This investigator in collaboration with Dr. McAdam established a RIA for mouse AA similar to the one which was employed for human AA (Ref. 5). We established that SAA is an acute phase reactant in the mouse (Ref. 4) and that this response can be induced by many antigenic and inflammatory stimuli. The only example we have found where SAA does not promptly rise after an acute phase stimulus is the C3H/HeJ strain of mice, which is unresponsive to the physiological effects of purified LPS. C3H/HeJ mice do respond to other acute phase stimuli with SAA elevation, as does the A/J strain which is resistant to induction of amyloidosis; thus production of SAA appears to be a normal phenomenon which occasionally may be involved with the development of a pathological state, amyloidosis (Ref. 4, 6).

Kinetic analysis of proteins related to the amyloid fibril protein AA during development of amyloidosis: The origin of AA and AA-related proteins during induction of amyloidosis in C3H mice has been investigated using the RIA for murine AA. The response of the amyloid resistant A/J strain of mice has been monitored to serve as a control for the non-amyloidogenic effects of the amyloid inducing agent, complete Freund's adjuvant with added Mycobacterium butyricum. The primary or preamyloid phase in C3H mice is characterized by an acute phase response to the amyloid-inducing agent in which the concentration of SAA is increased several hundred fold, and by a lag period in which SAA concentration is diminished as compared with acute phase levels, but still significantly elevated with respect to baseline concentrations (Ref. 6). The profile of SAA concentrations in A/J mice after treatment with the amyloid-inducing agents is similar to that of the C3H mice in which amyloidosis is induced.

The secondary, or amyloidotic, phase in C3H mice is marked by a thousand fold increase in the concentration of AA-related protein in the spleen. AA

proteins are found in the spleens of A/J mice much later, and then only in those instances where amyloidosis occurs. These findings are consistent with earlier morphological observations of many investigators which have given rise to the concept of the biphasic development of amyloidosis. The data also suggest that in the spleen, where the first amyloid deposits are detected, fibrils result from local cellular secretion. They further raise the question of whether SAA is actually the precursor of the AA fibril protein or is instead related to the polypeptide precursor of the AA protein through a common gene product.

Origin of SAA: The AA cross-reactivity in ng/mg wet weight of tissue was determined for liver, spleen, kidney, peritoneum, heart, lung, skin, fat, muscle, and whole animal homogenates during the early hours of the acute phase response to LPS. It was found that liver showed the earliest and largest rise in antigen content of all tissues. This elevation was parallel to that of plasma, and was significantly greater than that of spleen. The liver has been well documented as the tissue of C-reactive protein origin. The identical time response of these two acute phase reactants (CRP and SAA) in response to an inflammatory agent (Ref. 7) would suggest that there is a common method of induction for the two proteins and that SAA arises from de novo protein synthesis rather than from breakdown of connective tissue, which has been postulated by other workers.

Primary structure of proteins related to AA and non-AA amyloid proteins: It is not known what factors dispose a small percentage of patients with acute recurrent and chronic inflammatory to develop amyloidosis, since, as was mentioned above, all of them can be expected to produce elevated concentrations of SAA, and extremely high SAA concentrations can be induced in previously asymptomatic individuals. It may be that the primary structure of SAA in amyloidotic individuals predisposes it to formation of beta pleated sheet fibrils upon proteolysis. Other factors such as a defect in the lysosomal processing of SAA or synthesis of the AA precursor by an abnormal cell or tissue type may be involved. SAAL has molecular weight of 12,500 and would be expected to contain approximately 30 additional amino acid residues compared with the AA fibril protein which has molecular weights of 5500 to 9000. Other laboratories have shown that SAAL has the same primary structure at the amino terminus as AA and hence the additional amino acids would be expected to comprise an additional peptide segment at the carboxyl terminus.

In collaboration with Dr. Waxdal, this investigator developed microsequence methodology for amyloid proteins using the Beckman spinning cup protein sequencer and radioactive PITC. This methodology has been applied to the amyloid fibril protein associated with the familial Portuguese amyloid neuorpathy which is very closly related to normal serum prealbumin (Collaboration with Drs. Costa and Sobiech). The preliminary experimental results have suggested that there may be amino terminal heterogeneity of the Portuguese amyloid protein as well as internal similarity of CNBr derived peptides.

A Beckman microsensitivity amino acid analyzer was procured and installed for the purpose of carrying out primary structure determinations at the carboxyl portions of human amyloid and nonamyloid SAAL and murine amyloid susceptible and amyloid resistant SAAL by the subtractive Edman method.

Publications:


1. Linke, R.P., Sipe, J.D., Pollock, P.S., Ignaczak, T.F., and Glenner, G.G.: Isolation of a low-molecular-weight serum component antigenically related to an amyloid fibril protein of unknown origin. Proc. Natl. Acad. Sci. USA, 72, 1473-1476 (1975).

2. Sipe, J.D., Ignaczak, T.F., Pollock, P.S., and Glenner, G.G.: Amyloid fibril protein AA: Purification and properties of the antigenically related serum component as determined by solid phase radioimmunoassay. J. Immunol. 116, 1151-1156 (1976).

3. Sipe, J.D., McAdam, K.P.W.J., Torain, B.F., and Glenner, G.G.: Conformational flexibility of the serum amyloid precursor SAA. Br. J. Exp. Path. 57, 582-592 (1976).

4. McAdam, K.P.W.J., and Sipe, J.D.: Murine model for human secondary amyloidosis: genetic variability of the acute-phase serum protein SAA response to endotoxins and casein. J. Exp. Med. 144, 1121-1127 (1976).

5. Sipe, J.D., McAdam, K.P.W.J., Torain, B.F., and Pollock, P.S.: Isolation and structural properties of murine SAA-the acute phase serum precursor of amyloid AA. Immunol. Comm. 6, 1-12 (1977).

6. Sipe, J.D., McAdam, K.P.W.J. and Uchino, F.: Distribution of protein antigenically related to amyloid fibril protein AA in murine tissues and serum during induction of amyloidosis. Fed. Proc. 36, 3412 (1977).

7. McAdam, K.P.W.J., Elin, R.J., Wolff, S.M., and Sipe, J.D.: Serum amyloid A (SAA) and C-reactive protein (CRP) in acute inflammation. Fed. Proc. 36, 4180 (1977).

8. Ignaczak, T.F., Sipe, J.D., Linke, R.P. and Glenner, G.G.: Immunochemical studies on the nature of SAA and secondary amyloidosis. J. Lab. Clin. Med. 89, 1092 (1977).

9. Watanabe, S., Jaffe, E., Pollock, P.S., Sipe, J.D. and Glenner, G.G.: Amyloid AA protein: Cellular distribution and appearance. Am. J. Clin. Path. in press (1977).

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PERIOD COVERED

July 1, 1976 through September 30, 1977

TITLE OF PROJECT (80 characters or less)

Cytogenetics

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

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| Other: | B. J. White | Section on Cytogenetics | LEP NIAMDD |

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| G. Brecher | Univ. Calif. Medical Center, San Francisco |
| P. Tischler | Harvard Medical School, Boston |
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Cytogenetics

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SUMMARY OF WORK (200 words or less - underline keywords)

1) Application of differential staining techniques and continued improvement of established methods are basic to all of the projects conducted in the laboratory.
2) Human cytogenetic studies of patients with congenital and developmental disorders as well as studies of mutagen-induced chromosomal abnormalities are continuing.
3) Murine cytogenetic studies include evaluation of mitotic and meiotic chromosomes of Robertsonian translocation carriers and karyotypic evaluation of cells in hematopoietic tissue regeneration experiments. Immunogenetic and cytogenetic characteristics are also being studied in NZB and DBA mice. These are part of projects utilizing the mouse as a model for human diseases.



PHS-6040

Project Description:

Cytogenetics of Hemtopoetic Disorders.

In collaboration with Dr. Germain (university Claude-Bernard, Lyon, France), cytogenetic studies have been carried out using the sister chromatid exchange (S.C.E) frequencies as a criterion to compare chromosomal alterations found in (1) constitutional hemopathies associated with leukemia or preleuke[illegible], and (2) abnormalities of the hematopoetic system induced by leukemogenic substances such as benzene and related polycyclic hydrocarbons with normal, healthy subjects as controls.

The results of these studies are being worked out.

Experiments on Pluripotential and Committed Hemopoietic Stem Cells.

As a joint project with Dr. G. Brecher of the Department of Laboratory Medicine, University of California at San Francisco, experiments are being conducted to test a new hypothesis concerning the kinetics of pluripotential stem cells (PSC). It is based on the assumption that the pluripotential stem cells are normally not in cycle. The implications of this thesis are that (1) CSC (committed stem cells) compartments are largely self-maintaining and capable of meeting transient demands for increased hemopoeisis, (2) non-cycling PSC explain the failure of normal marrow to take in syngeneic recipients, and (3) "turning on" the PSC will permit seeding normal syngeneic recipients with normal marrow.

In order to verify this hypothesis, a series of experiments have been conducted in which we have been able to show that normal PSC survive in normal recipients, at least for a day, and may proliferate massively. Bone marrow from female C57B1 and C3H mice was infused into isogeneic recipients of the opposite sex, which were exposed to a uniformly lethal dose of X-irradiation one hour later, with the liver and spleen shielded. By using the C-banding technique, the male and female metaphase cells can be identified in the spleen and marrow of the host animals. Experiments are now under way to determine how long after transfusion the normal cells can be induced to proliferate, whether modalities other than X-ray many "turn on" the transfused cells, and whether cells turned on in the donor could proliferate on subsequent transfusion into normal hosts.

Meiotic Chromosomes of the Female Mouse.

This study will be continued. Findings, thus far obtained, of the rate of meiotic nondisjunction in female mice heterozygous for three Robertsonian translocation chromosomes are very similar to those observed in the male meiosis. This was also the case in G.P. mice and in our 34 chromosome strain. Experiments will be carried out to determine which factors may cause or play a role in inducing nondisjunction during anaphase I and/or anaphase II (i.e. age, heterozygous conditions, viral infection, X-ray, and chemicals).

Fluorescent In Situ Hybridization for Gene Mapping.

This study is a collaborative project with Dr. P. Tischler, Harvard Medical School, Boston and Dr. B. Forget, Yale Medical School, New Haven. Experiments will be carried out to validate a new method for mapping genes on

chromosomes. This method, developed in Dr. Tischler's laboratory, involves the in situ hybridization of the poly-adenylic acid tail of the RNA to a fluorescent sphere containing poly-uridelic acid. The technique will be used to study the mouse globin genes since these have been mapped by conventional techniques. Metaphase chromosome preparations are initially stained with quinacrine mustard (Q.M.), and each chromosome identified. These same chromosomes are then hybridized to the messenger RNA and the fluorescent spheres. They are then re-examined by fluorescent microscopy, and the chromosomal location of the fluorescent spheres are noted. We anticipate that the spheres will detect $\alpha$-globin messenger RNA bound to chromosome 7 and $\beta$-globin messenger RNA bound to chromosome 11.

Clinical Cytogenetic Studies of Human Congenital and Developmental Disorders.

Patients referred to our laboratory for karyotypic diagnosis are those with various unusual genetic disorders, abnormalities in gonadal development or function, and selected cases of infertility in the male. In these types of cases, we are looking for minor variants or abnormalities as well as gross morphological changes. With routine karyotyping many of these anomalies could be overlooked or misinterpreted; therefore, all preparations from such patient referrals are Giemsa- and quinacrine-banded. Additional procedures such as centromeric-banding, sister chromatid exchange, and/or late replication studies are used if a chromosome abnormality is found or suspected. With the addition of new and improvement of our older, more standard banding procedures, we are now better prepared to detect karyotypic variations and more capable of correctly interpreting them.

Murine Immunogenetic and Cytogenetic Studies.

Experiments are currently being performed in collaboration with Dr. A. Steinberg (NIAMDD) to determine the effects of the NZB genetic background on the immunological and cytogenetic status of the NZB/DBZ $F_1$ hybrid and backcross mice. The NZB strain is the mouse model for the human autoimmune disease, lupus erythematosus. Direct chromosome preparations of NZB female spleens have shown that all adult females tested have aneuploidy; this was not the case in direct chromosome preparations of the bone marrow. In contrast, NZB male spleen direct chromosome preparations showed little, if any, aneuploidy. $F_1$ hybrid mice of both sexes also showed a normal chromosome complement in both the bone marrow and the spleen. Backcross animals are currently being investigated for the presence of aneuploidy. Thus far, only backcross females produced by crossing $F_1$ hybrids with NZB mice have shown any aneuploidy. Further investigations are under way to determine if this chromosomal instability is a genetically inherited trait and what role hormones play in influencing this instability.

These same mice have also been studied for immunological abnormalities. The parent NZB strain produces autoantibodies which are cytotoxic for thymocytes. NZB/DBZ $F_1$ females in 50% of the cases had a positive titer of naturally occurring thymocytotoxic antibodies, while intact males had a 0% incidence. Castration of the males increased the incidence of thymocytotoxic antibodies to the level of their female littermates, but removal of the ovaries from female $F_1$ hybrids did not alter the response. Backcross animals showed a similar sex distribution. Backcrosses to the NZB parent had a higher

incidence of thymocytotoxic autoantibodies than did the $F_1$ hybrids, but a lower incidence than the NZB parent. Backcrosses to the DBA parent had a lower incidence of thymocytotoxic autoantibodies than did the $F_1$ hybrids, but a higher incidence than the DBA parent.

Both the immunologic and cytogenetic abnormalities seen in NZB mice and their hybrids seems to be under genetic and hormonal control.

Publications:

Raveche, E. S., Klassen, L. W., and Steinberg, A. D.: Sex differences in formation of anti-T-cell antibodies. Nature 263: 415-416, 1976.

Brecher, G., Tjio, J. H., Smith, W. W., and Haley, J. E.: Marrow regeneration after mechanical depletion. Blood 48: 679-686, 1976.

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Cytogenetic Studies

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

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INSTITUTE AND LOCATION

NIAMDD, NIH, Bethesda, Maryland 20014

| TOTAL MANYEARS: | PROFESSIONAL: | OTHER: |
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CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

1) Cytogenetic and reproductive studies of murine Robertsonian translocation carriers. Analysis of adult and fetal somatic and gonadal tissues using chromosomal banding techniques in order to study meiotic nondisjunction and characteristics of aneuploid progeny from translocation heterozygotes.

2) Cytogenetic and dermatoglyphic analysis of patients with congenital defects, recognized dysmorphic syndromes, abnormal gonadal development and function, and other genetic disorders. Techniques used for cytogenetic studies include Giemsa-, quinacrine-, and centromeric-banding, as well as fluorescent-Giemsa analysis of chromosomal replication sequences. Cytogenetic, dermatoglyphic, and other clinical studies are correlated.

3) Karyotypic evaluation of potentially abnormal tissues. These studies utilize a variety of chromosomal banding methods for analysis of tissues from laboratory animals (primate and murine) suspected of having chromosomal abnormalities. Relationship of cytogenetic findings to genetic background, development of tumors, and reproductive fitness is emphasized.

Project Description:

1) Cytogenetic and reproductive studies of murine Robertsonian translocation carriers.

Our studies of a series of laboratory mouse Robertsonian translocations have continued. One of the original strains Rb(5.19), now in generation 39 of inbreeding, is now being used by three groups of investigators for cleft palate studies. So far, they have demonstrated that palatal horizontilization and growth are delayed in this strain, and that it is highly sensitive to cortisone-induced cleft palate. This "sensitivity", correlated with the H-$2^a$ genotype in the Rb(5.19) and another sensitive strain (A/Jax), is associated with increased palatal cytosol binding for cortisol. Resistant strains such as CBA have less binding. These results may in part explain our earlier observations of cleft palate in fetuses with translocation trisomy 19 detected in crosses of Rb(5.19) x Rb(9.19) $F_1$ hybrids. The palates of chromosomally normal littermates were intact, suggesting that trisomy 19 was the major predisposing genetic factor. However, other autosomal loci not on chromosome 19 appeared to influence cleft palate, since it was more frequent in trisomics from backcrosses to Rb(9.19) than to Rb(5.19), and not present in trisomics from outcrosses to NIH GP mice. In the future, we hope to determine if the H-2 genotype of susceptible trisomy 19 fetuses correlates with the H-$2^a$ genotype of the Rb(5.19) parental strain.

Our most recently established combined translocation strain, Rb(5.19)/(6.15)/(8.17), has now been randomly bred since 1974. Direct meiotic studies of male homozygotes, who carry three pairs of bi-armed translocation submetacentrics, have shown a very low frequency of abnormal meiotic disjunction. Both male and female $F_1$ heterozygotes of this strain, obtained by outcrosses to NIH GP, have now been studied. Both have frequent abnormal meiotic disjunction due to irregular segregation of three trivalents associated with the three translocations. Both male and female $F_1$ have frequent trisomic embryos in backcrosses with NIH GP mice. Our most recent studies show that males give significantly more trisomic embryos than females, even though their cytological studies indicate similar frequencies of nondisjunction. While this discrepancy could be related to technical problems in the meiotic chromosome analysis, other investigators noting similar discrepancies have suggested that it could have a genetic basis. It has even been proposed that survival of chromosomally abnormal gametes and pre-implantation embryos may be influenced by H-2 genotype or other gene complexes on chromosome 17. At present, we are considering exploring the association between these genetic markers and our observations that viability and phenotype of trisomic embryos is strain-influenced. A manuscript concerning establishment and meiotic disjunction of the Rb(5.19)/(6.15)/(8.17) strain is in preparation.

2) Cytogenetic and dermatoglyphic studies of patients.

These studies of a limited number of patients with congenital defects, genetic syndromes, and abnormal gonadal development have continued. Cases were selected so that either relationship of clinical, chromosomal, and dermatoglyphic findings could be ascertained, or so that specific types of structurally abnormal chromosomes could be studied with differential staining techniques. The latter include Giemsa-, quinacrine-, and centromeric-banding. The BUdR-Hoechst 33258 technique was utilized to study replication patterns

of normal X chromosomes, as well as to identify replication sequences in sex chromosomes of ambiguous origin. In several cases cytogenetic analysis was correlated with results of testing for H-Y cell surface antigen. The latter test, currently being done by Dr. Stephen Wachtel of Sloan Kettering, is of particular value in determining the presence of Y-linked, male determining genes when conventional cytogenetic techniques fail to detect a typical Y chromosome, and clinical findings suggest one should be present. In our cases (one 46,XX true hermaphrodite and two gonadal dysgenesis patients with non-fluorescent Y chromosomes) a positive test has been correlated with presence of testicular elements. Case reports of the latter patients are planned, and one case report concerning correlation of cytogenetic, dermatoglyphic, and radiographic findings in pseudohypoparathyroidism is being submitted for publication.

Short Stature: During a two-year period we have screened all female short stature referrals to the NNMC Pediatric Endocrinology Unit for sex chromosome anomalies in collaboration with Dr. Roger Johnsonbaugh. A total of 8 abnormals were identified during that time, giving a frequency of 29.6% of defects such as 45,XO, 45,XO/46,XX, and 45,XO/46,XY. These results were presented at a military medical meeting by Dr. Johnsonbaugh, and at present we are in the process of determining whether to continue the study or to report the present results. So far, they indicate that chromosome analysis is definitely indicated in females with short stature when: (1) other family members are of normal stature and, (2) when no other etiology is apparent.

We are also continuing our dermatoglyphic and cytogenetic studies of patients with Kallman's syndrome (hypogonadotropic hypogonadism with anosmia). So far, chromosome studies of 15 cases have been normal, as expected. Dermatoglyphic analysis of the first 13 cases shows: (1) frequent complete or bridged simian crease (5 of 13), (2) increased mean summed atd angles, greater in males than females, due to increase in frequency of intermediately distally displaced axial triradii, and (3) decreased frequency of arches and ulnar loops, compensated for by an increase in a particular transitional pattern type (central pocket whorl). A-B ridge counts were unaffected, and the number of patients was too few to determine if total finger ridge count was altered. This project will be continued as long as cases become available.

Another dermatoglyphic project in progress is analysis of 25 sets of twins with multiple sclerosis, in collaboration with NINDS. Disease is concordant in some of the identical twin pairs. Almost all of the sixteen individuals printed so far have moderate or marked dermal ridge atrophy, except for two unaffected fraternal twins.

3) Karyotypic evaluation of potentially abnormal tissues.

Murine Tumors: Our studies of AKR thymomas during the past two years with Dr. W. Rowe (NIAID) indicated that the aneuploidy found in most of these tumors is not autosome specific. However, we have noted frequent monosomy X in tumor cells from female hosts, a finding reported by other investigators in other types of murine tumors. Because of improvement of our analysis technique, we were able to determine that those tumors with normal chromosome counts (2n = 40) actually had abnormal "pseudodiploid" karyotypes. Initially, the search for specific aneuploidy was initiated as part of an attempt to map the AKV-2 locus associated with tumorigenesis. Our results indicate that,

in this case, cytogenetic analysis is not a useful technique for gene mapping. From our work, there is no evidence that presence of a genetic locus associated with tumor susceptibility predisposes that particular chromosome to become involved more frequently in the nondisjunctional process resulting in aneuploidy. This study is not yet completed.

Primate Chromosomes: We are continuing studies of a series of chromosomal inversions in squirrel monkeys in collaboration with Dr. J. Newm NICHD. At present we are attempting to determine if inversion heterozygosity affects fertility and infant survival. Histological studies of the gonads of several stillborn animals are being done in order to compare gonadal development of chromosomally normal and abnormal monkeys. This long-term study, in which Dr. Newman is correlating behavioral and genetic factors, may also provide information about one of our greatest interests, the effects of chromosomal imbalance upon germ cell development.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)

U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE
PUBLIC HEALTH SERVICE
NOTICE OF
INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER
ZOI AM 21,010-01 LEP

PERIOD COVERED
July 1, 1976 through September 30, 1977

TITLE OF PROJECT (60 characters or less)

Function of DNA virus genomes in animal cells

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

| | |
|---|---|
| Barrie J. Carter | Senior Staff Fellow |
| Luis M. de la Maza | Visiting Associate |
| Catherine A. Laughlin | Staff Fellow |
| Maureen W. Myers | Staff Fellow |
| Francis T. Jay | Visiting Fellow |
| Hiroshi Handa | Visiting Fellow |

COOPERATING UNITS (if any)
William J. Cook (C:LP), K. Berns and K. Fife, Johns Hopkins University; B. Moss, NIAID; W. Doerfler, Institute of Genetics-Cologne; H. Westphal, NICHD; D. Ward, Yale.

LAB/BRANCH
Laboratory of Experimental Pathology

SECTION
Section on Chemical Pathology

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland

| TOTAL MAN-YEARS: | PROFESSIONAL: | OTHER: |
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CHECK APPROPRIATE BOX(ES)

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

We are employing DNA viruses as molecular probes to study genome expression in human cells. We are studying intensively the structure and function of adeno-associated virus (AAV) since this virus has only a single gene. We are studying also adenovirus which is a more complex genome. Adenovirus is the helper virus for AAV multiplication. This helper relationship is being analysed. Also both AAV and adenovirus recombine with cellular DNA. In the case of adenovirus this causes malignant transformation of the cell. AAV can inhibit this transformation and also inhibits Ad12 oncogenesis in newborn animals. Thus AAV inhibits tumor induction. The mechanism of this inhibition of tumor induction is being studied at the molecular level in both cell culture and in animal experiments.

Project Description:

Adeno-associated viruses (AAV) are defective parvoviruses. Because of the small size of their DNA genomes and unusual mode of segregation of complementary DNA strands to separate virions, the AAV offer a unique situation amenable to biochemical analysis. AAV synthesizes only a single mRNA species (for its coat protein). Thus the AAV genome seems to consist of a single unique gene enclosed by DNA regions containing reiterated sequences. We are studying AAV as a model system for eukaryotic gene expression. The main objectives of this study are: to analyse the structure of AAV DNA and obtain an accurate transcription map; to study the control of synthesis, post-transcriptional processing and modification and transport and degradation of AAV; to study interactions between AAV and its helper adenovirus in both permissive and non-permissive cells. In order to analyse the interaction between AAV and adenovirus we require additional information directly relating to adenovirus function in infected cells. We have therefore initiated studies on various parameters of the adenovirus growth cycle.

Major findings since the last report are:

Structural Analysis of AAV DNA: Duplex AAV-2 DNA was digested with SalI, PstI, or HaeII restriction endonucleases and the cleavage sites were mapped. SalI cleaves AAV DNA at 0.310 map units, PstI at 0.106, 0.422, and 0.914 and the five HaeII sites were mapped at 0.110, 0.156, 0.181, 0.536, and 0.600 map units. These cleavage products will be useful for the isolation of specific regions from the AAV DNA, located outside of the stably transcribed region of the genome, and also helped to map more complex restriction enzyme cleavages.

Linear duplex molecules of adeno-associated virus type 2 (AAV2) DNA were cleaved with the restriction endonucleases R.HhaI, R.HaeIII, R.HpaII, and R.AluI each of which produced about 20 fragments. These fragments were analysed by electrophoresis in polyacrylamide gel slabs and their physical order on the AAV genome map was determined. This work provides a map of approximately 75 additional cleavage sites on AAV DNA. The inverted terminal repetitious sequence was estimated to be 80 to 120 nucleotides. The results obtained, considered together with previous data, are inconsistent with the model of two permutations of AAV DNA strands having different startpoints. Rather, it appears that the "permutation" in AAV DNA results from an unusual secondary structure located within the inverted repetition, between 20 and 100 nucleotides from the terminus.

The total restriction map of AAV now provides more than 100 cleavage sites which have been accurately mapped. This allows very precise mapping of potential control regions such as transcription promoters or terminators. It has also allowed characterization of incomplete or variant molecules of AAV (see below).

Structure of the DNA from Incomplete AAV Particles: Adeno-associated virus-2 (AAV-2) particles containing incomplete genomes were separated from those containing complete viral genomes in cesium chloride density gradients and/or sucrose velocity sedimentation. The incomplete particles banded in the CsCl isopycnic centrifugation at densities of approximately 1.40 and 1.36-1.33

g/cc. Incomplete particles with a density of 1.40 g/cc yielded two main types of DNA (types I and II) having molecular weights of $1.4 \times 10^6$ and $0.8 \times 10^6$ respectively in both neutral and alkaline pH. As assayed with S1 nuclease, at least 70% of the type I and II molecules are cross-linked and probably represent an inverted repetition of a restricted portion of the AAV genome. Restriction endonuclease cleavage with EcoR1, Alu, HhaI, HpaII, HaeIII, and HaeII showed that type I molecules contain 35-50% and type II contain 20-30% of either the left-hand end or right-hand end of the AAV genome. Both "permutations" of the terminal restriction fragments obtained from complete AAV DNA were also present in the incomplete molecules. Incomplete DNA molecules from particles banding at a density of 1.36-1.33 g/cc (type III) had single strand molecular weights of $0.2\text{-}0.6 \times 10^6$. These molecules contained up to 20% of the genome sequences from one or both termini and both terminal "permutations" were present. Type III molecules appear to be a mixture of both internally deleted molecules and hairpin structures analogous to type I and II. The smallest incomplete molecules contained only 3-5% of the terminal region of the complete genome. The heterogeneous size of incomplete molecules is due to variations in the amount of the internal region of the AAV genome present. Thus restriction enzyme cleavage results in a gradient of fragment concentrations such that terminal fragments are represented in highest yield and those furthest from the termini in lowest molar yield. Since every molecule appears to contain an AAV genome terminus which is believed to be the site of the origin of DNA replication, then at least the snap-back incomplete molecules may arise from aberrant DNA replication rather than by a recombinational mechanism.

These incomplete molecules are now being used to analyse the mechanism of AAV replication as well as adenovirus replication. More importantly, these incomplete molecules have turned out to be very useful probes for analysing the mechanism of the inhibition by AAV of oncogenesis mediated by adenovirus type 12 in newborn hamsters.

AAV RNA Transcription: We have continued our studies on transcription of AAV mRNA *in vivo* using human KB cells infected with AAV and Ad2. We have also initiated *in vitro* studies on AAV transcription in an attempt to reconstruct the cellular transcription machinery.

Previous studies, which showed that stable AAV RNA synthesized *in vivo* anneals only to the DNA minus strand between 0.18 and 0.88 map units, may not have detected unstable or nascent RNA. Therefore, AAV-infected KB cell lysates were fractionated by high salt precipitation into a pellet containing nascent (chromatin-associated) RNA and a supernatant containing non-chromatin associated RNA. AAV RNA sequences in each fraction were hybridized with separated strands or restriction fragments of viral DNA. Supernatant RNA contained the same sequences as the previously mapped stable RNA. Chromatin-associated RNA contained the same sequences as supernatant RNA as well as sequences mapping to the left (upstream) of 0.18 and to the right (downstream) of 0.88 map units. Neither RNA fractions contained sequences complementary to the plus DNA strand. Thus, AAV RNA transcripts may be cleaved at the 5' end prior to methyl capping and at the 3' end prior to polyadenylation.

We have begun analysis of AAV RNA synthesis in an *in vitro* system derived by sarkosyl lysis of infected cell nuclei in 0.1 M salt. This

released at least 50% of the AAV viral transcription complex (VTC) but little, if any, cellular or adenovirus transcription activity. Solubilized complexes required all 4 nucleoside triphosphates and $Mn^{++}$, were maximally active at 23°C, and were completely inhibited by actinomycin D or α-amanitin at 2 μg/ml. Thus AAV RNA is synthesized by RNA polymerase II. Since the VTC activity was insensitive to heparin, RNA chain initiation did not occur. The VTC sedimented in sucrose gradients at about 16-20S on the leading edge of the AAV replicating molecules. A second VTC peak at 10S may be complexes in which incomplete AAV molecules are the template.

AAV DNA was transcribed in vitro with purified KB cell polymerase II. Denatured DNA was twice as efficient as native DNA as a template. RNA from both templates was about 30% resistant to ribonuclease digestion. Also, separated plus or minus strands of AAV DNA were equally good templates. These findings indicate that transcription was symmetric. The RNA synthesized was heterogenous in size but some nearly full length transcripts were present.

Publications:

B. J. Carter. (1976). Intracellular distribution and polyadenylate content of adeno-associated virus RNA sequences. Virology 73, 273-285.

B. J. Carter, K. H. Fife, L. M. de la Maza, and K. I. Berns. (1976). Genome localization of adeno-associated virus RNA. J. Virol. 19, 1044-1053.

L. M. de la Maza and B. J. Carter. 1976. Cleavage of adeno-associated virus DNA with SalI, PstI and HaeII restriction endonucleases. Nucleic Acids Res. 3, 2605-2616.

B. J. Carter, L. M. de la Maza, and F. T. Jay. (1977). The structure of the adeno-associated virus genome. In "DNA Insertion Elements: Plasmids and Episomes" (Eds., A. I. Bukhari, J. Shapiro and S. Adhaya), Cold Spring Harbor, New York. in press.

L. M. de la Maza and B. J. Carter, 1977. Adeno-associated virus DNA structure. Restriction endonuclease maps and arrangement of terminal sequences. Virology, in press.

B. J. Carter. (1977). Parvovirus Transcription. In "Parvoviruses: Replication and Biological Properties" (Eds., D. Ward and P. Tattersall), Cold Spring Harbor, New York, in press.

F. T. Jay, C. A. Laughlin, W. J. Cook, L. M. de la Maza and B. J. Carter. (1977). Adeno-associated virus RNA synthesis in vivo and in vitro. In "Parvoviruses: Replication and Biological Properties" (Eds., D. Ward and P. Tattersall), Cold Spring Harbor, New York, in press.

L. M. de la Maza and B. J. Carter. (1977). DNA structure of incomplete AAV particles. In "Parvoviruses: Replication and Biological Properties" (Eds., D. Ward and P. Tattersall), Cold Spring Harbor, New York. in press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)

U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE
PUBLIC HEALTH SERVICE
**NOTICE OF INTRAMURAL RESEARCH PROJECT**

PROJECT NUMBER

ZOI AM 21,011-01 LEP

PERIOD COVERED

July 1, 1976 through September 30, 1977

TITLE OF PROJECT (80 characters or less)

Investigation into boundary lubrication

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

James F. Wilkins Staff Fellow

COOPERATING UNITS (if any)

None

LAB/BRANCH

~~Laboratory of Experimental Pathology~~

SECTION

Section on Cellular Function and Ultrastructure

INSTITUTE AND LOCATION

NIAMDD, NIH, Bethesda, Maryland

| TOTAL MANYEARS: | PROFESSIONAL: | OTHER: |
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SUMMARY OF WORK (200 words or less - underline keywords)

Experimental work has been done on the molecular requirements for boundary lubrication, including investigation into molecular analogs of hyaluronate protein. The molecular mechanism for boundary lubrication has also been studied concerning the lubrication of the sliding joints of the skeleton.



PHS-6040
(Rev. 10-76)

Project description:

Molecular Requirements for Boundary Lubrication: Chemical synthesis of a number of different molecular analogs of the putative biological boundary lubricant, hyaluronate-protein, was tried using macromolecular polycarboxylic acids and globulin proteins coupled with carbodiimides. None lubricated in the friction testing apparatus. This negative result has many possible interpretations that are unresolved. Had the attempt succeeded, the synthetic system would have allowed selective manipulation of components in order to clarify the molecular requirements for boundary lubrication.

Boundary Lubricating Films: An expected third feature of natural boundary lubricating films adsorbed from synovial fluid onto articular cartilage has been considered. The inherent tendency not to adhere to the apposed and identical film only about 10 nm removed, helps the hyaluronate deposit resist the large shearing forces developed in joint movement, thereby preserving its integrity.

Molecular Mechanism for Boundary Lubication: A controversial view of the molecular mechanism involved in boundary lubricating the sliding joints of the skeleton is being studied.

Anchoring of hyaluronate-protein to the adsorbing surface through tight adhesion of the protein component. Without this firm attachment, the cartilage surface can be stripped of lubricant by shearing forces generated during joint movement.

The adsorption of hyaluronate-protein, with its many carboxylate groups, increases the area concentration of negative electrical charges on the cartilage surface. Two such charged surfaces mutually repel, and when pressed together by forces typical of joint loading, they cannot approach one another closer than about 100 Å. This gap, filled with physiological electrolyte, can provide a viscous drag "friction" in the 0.01-0.02 range commonly observed for boundary lubrication by synovial fluid, as long as at least one of the rubbing surfaces is soft, and able to ride over the asperities in the mating surface in order to maintain the thin 100 Å fluid layer.

The associated electrostatic floatation calculations need more work. Surface potentials of 50-80 millivolts are expected for articular cartilage covered with adsorbed hyaluronate-protein. Such values are biologically reasonable. Mathematical treatment is complicated by the inapplicability of the usual Debye-Huckel theory to surface potentials greater than 25 mv.

Adsorbed hyaluronate-protein has an additional property, that of nonseizure with similar surfaces, which is a seemingly general characteristic of dilute hydrogels. This effect discourages cross-joint entanglement and locking of the absorbed lubricant film pair. Tentative local microseizures across the sliding joint should separate at the coupled gel's outer loops with very little film damage, as a consequence of weakest link breakage.

Synthetic Synovial Fluid: The 4th area of research concerns adsorbed, high molecular weight polyacrylic acid's successfully imitating synovial fluid's

boundary lubrication of rubber sliding on glass. Polyacrylic acid is one of the few compounds to do this. In contrast to hyaluronate-protein, it is a relatively simple molecule, without biochemical contaminants. Physical chemists have studied it extensively as a model for polyelectrolyte behavior.

LABORATORY OF CHEMICAL BIOLOGY, NIAMDD
ANNUAL REPORT: JULY 1, 1976 - SEPTEMBER 30, 1977

As in previous years, the major effort of the Laboratory of Chemical Biology is concerned with the chemistry, function and three-dimensional conformation of macromolecules. A somewhat greater effort has been put on the application of immunological methods in the studies of aggregative systems, such as sickle cell hemoglobin, to the studies of conformation of proteins and nucleic acids and of the genetic regulation of their synthesis and to the use of specific antibodies as tools in purification of proteins by immunoabsorbance affinity chromatography. Considerable progress has also been made for the preparation of larger amounts of human interferon, now beginning to be available in amounts adequate for initial structural studies.

## I. Chemistry, function and three-dimensional conformation of macromolecules

### A. Studies of gelation of sickle hemoglobin: peptides as possible inhibitors

To investigate the antisickling potential of various agents on deoxygenated hemoglobin S, we have used the assay developed by Hofrichter, Ross and Eaton which monitors gelation kinetics via turbidity changes at 800 nm and measures solubility using ultracentrifuge sedimentation. The antisickling effect as measured by this assay has been determined for the amino acids, some dipeptides and several aromatic compounds. The aromatic amino acids, and phenylalanine analogs have been found to inhibit the gelation of deoxyhemoglobin S and hence may indicate a potential class of therapeutic anti-sickling agents. This project will be expanded to other compounds including oligopeptides in a continuing effort to study the surface interactions of deoxyhemoglobin S. (Noguchi and Schechter)

### B. Monospecific antibodies as probes of hemoglobin structure and function and the binding of hemoglobin to red cell membranes

Using non-precipitating monospecific antibodies as probes of conformation, we are studying structural differences of hemoglobins in solution. Hemoglobin $A_o$ and $A_2$ have been isolated, purified and carbamoylated with $K^{14}CNO$. Two specific subpopulations of anti-hemoglobin antibodies have been isolated by affinity chromatography using synthetic peptides corresponding to $\alpha(129\text{-}141)$ and $\delta(5\text{-}13)$. The first subpopulation will be used to study conformational changes at the carboxyl terminus of the $\alpha$ chain during oxygenation based on the difference in the binding affinity between $^{14}$[C]-oxy and $^{14}$[C]-deoxy hemoglobin. Differences will be used to examine mutant and truncated hemoglobins so as to better understand the structural changes occurring during the dynamic transition from the T (deoxy) state to the R (oxy) state. The second subpopulation will b used to measure hemoglobin $A_2$ levels in serum and in individual cells and to differentiate $A_2$ from other normal and mutant hemoglobins. In addition we are using the labeled normal hemoglobins to quantitate the binding of hemoglobin to red cell membranes and to determine the site of this binding. (Dean, Eastlake-Dean, Schechter)

C. Immunological and structural studies of human fetal Hb

Human fetal hemoglobin (HbF) differs from HbA in both functional and structural aspects. These differences are evidenced by the weaker affinity of HbF for 2,3 diphosphoglyceric acid, and by the non-participation of HbF in gel formation with HbS. The molecular basis of these differences is not clear. There are 39 amino acid differences between the $\gamma$ chains of HbF ($\alpha_2\gamma_2$) and the $\beta$ chains of HbA ($\alpha_2\beta_2$) several of which are found in the 2,3 diphosphoglyceric acid binding site. A number of other amino acid substitutions are clustered in one exposed helical region of the $\alpha$ chain. We are using solid phase synthesis to reproduce this helical region. Immunoabsorption with this synthetic peptide of an antiserum to HbF is being used in an attempt to obtain non-precipitating antibodies specific for this region which do not crossreact with HbA or other hemoglobins. A radioimmunoassay using [$^{14}$C]-carbamoylated HbF will be used to assess the specificity of binding. We have also obtained crystals of met-HbF of suitable size for X-ray diffraction studies. The specific antibodies and X-ray studies will give information on the conformational and surface structural characteristics of HbF. In addition the antibodies may be useful in quantitating small amounts of HbF in standard laboratory procedures and in prenatal diagnosis of hemoglobinopathies. (Eastlake-Dean and Schechter)

D. Physical chemical studies of self assembling protein systems: hemoglobin and tubulin

The rotational motion of sickle hemoglobin in gels has been monitored by $^{13}$C NMR with new decoupling procedures as a probe of the gelation process upon deoxygenation. It has been possible to quantitate the degree of immobilization in gels formed from cell-free hemoglobin solutions, as well as the intracellular gelation or sickling process. In addition, theoretical analysis of the results of recent inhibition studies of the gelation amino acids indicate that the inhibitor may be due to direct, but very weak, binding between amino acids and hemoglobin.

Detailed theoretical analyses of the disagreement between calorimetric and van't Hoff enthalpies of tubulin polymerization cast doubt upon the interpretation of many in vivo microtubule assembly studies. These analyses may be extended to include kinetic aspects of sickle hemoglobin gelation. (Sutherland and Schechter)

E. Studies of the mechanism of renaturation of proteins containing disulfide bonds after reduction and oxidation

Reduced partially carboxymethylated hen egg white lysozyme (approximately 0.8 mol of [1-$^{14}$C] carboxymethyl groups) was air oxidized at pH 8.0, 37°C in the presence of 1.5 mM $\beta$-mercaptoethanol for 36 hours. The material thus obtained was fractionated by gel filtration into the lower (native) and higher hydrodynamic volume forms. Both these fractions contained radioactivity, approximately 35 and 65%, respectively. Ion exchange chromatography of the lower hydrodynamic volume forms yielded renatured lysozyme, two major radioactive samples ($LH_C$ and $LH_D$) eluting at the positions of monocarboxymethylated lysozyme and two minor radioactive samples eluting at the positions of dicarboxymethylated lysozyme. Sample $LH_C$ (approximately 23% of the

radioactivity) was essentially homogeneous with respect to electrophoretic mobility, exhibited approximately 35% of the enzymatic activity and contained 0.95 mol of $^{14}C$-carboxymethyl groups. Sample $LH_D$ (approximately 8% of the radioactivity) was also enzymically active and contained approximately 0.5 mol of $^{14}C$ carboxymethyl groups, this low value is apparently due to contamination of non carboxymethylated species. The radioactive tryptic peptides from samples $LH_C$ and $LH_D$ were characterized. The results indicated that all the eight isomers containing three, presumably native disulfide bonds and one free and one carboxymethylated sulfhydryl group are formed during air oxidation of the reduced partially carboxymethylated lysozyme. Since in each one of these isomers the formation of one of the four native disulfide bonds is permanently blocked, it would follow that no one of the four disulfide bonds of native lysozyme is obligatory in the formation of other three native disulfide bonds. (Acharya and Taniuchi)

F. Studies on the structure-function of cytochrome c using a fragment complementing system

A heme containin fragment of cytochrome c consisting of amino acid residues 1 through 38 (1-38H) was prepared by tryptic digestion of the native molecule. Apocytochrome c (1-104) has also been prepared by removal of the heme moiety through standard chemical techniques. On mixing these two fragments a non-covalently linked 1:1 complex is formed, and the spectral properties and biological activity of this complex are essentially identical to native cytochrome c. The redundant residues of this native-like complex were removed by tryptic digestion of the material in its reduced form ($Fe^{+2}$). Subsequent separation and identification of the remaining complimenting fragments revealed that two distinct complexes are formed from the initial mixture of (1-38H) and apoprotein. Thus one complex consists of residues (1-25H) from the original (1-38H) fragment and residues (23-104) from the apoprotein, while a second complex utilizes the entire (1-38H) fragment and residues (56-104) from the apoprotein. In addition, we have shown that these native-like species may be regenerated by mixing the appropriate non-overlapping fragments. Biologically active complexes have been prepared from mixtures of (1-25H) + (23-104) and (1-38H) + (56-104) as well as (1-38H) + (39-104). (Parr and Taniuchi)

G. Protein unfolding by fragment exchange with a system derived from cytochrome c

The (1-53)H:(54-104) complex, an ordered complex formed by non-covalent interactions of the two fragments of cytochrome c formed by a limited trypsin digestion of the parent complex (1-65)H:(1-104), has been used in fragment exchange experiments to determine the activation free energy for unfolding of both oxidized and reduced forms of the complex. The slower rate of exchange of the ferrous form is consistent with its lack of susceptibility to trypsin digestion. The data support the concept of a dynamic equilibrium of folding and unfolding of cytochrome c under physiological conditions. (Hantgan and Taniuchi)

H. Studies on the dynamics of protein conformation

Precession photographs of crystalline Nuclease-T' are being taken at many levels of reciprocal lattice planes and the reflections thus recorded are being processed using a computer in order to obtain a difference electron density map between nuclease A and Nuclease-T.

The extra amino acid sequence contained in nuclease B, a possible precursor of nuclease A, has been established to be: Ser-Glu-Thr-Asp-Asx-Gly-Val-Asx-Arg-Ser-Gly-Ser-Glu-Asp-Pro-Thr-Val-Tyr-Ser where the two residues indicated by Asx are Asp in nuclease B1, and Asn in nuclease B3 and the first and the second Asx from the $NH_2$-terminus are Asp and Asn, respectively in nuclease B2. This extra sequence attached to the $NH_2$-terminus of nuclease A is flexible and does not interfere with the ordered structure or the function of the nuclease A portion.

Saturation of Nuclease-(1-126) with ligands ($pdT_p$ and $Ca^{2+}$) appears to decrease its intrinsic viscosity from 6.59 to 5.28 $cm^3/g$. This value for the intrinsic viscosity of presumably liganded Nuclease-(1-126) is equivalent to that of liganded nuclease B and clearly greater than that of liganded nuclease A. These results are compatible with the idea that liganded Nuclease-(1-126) may possess a conformation composed of ordered as well as flexible segments due to lack of cooperativity. (Taniuchi, Parker and Davis)

I. Functional and physicochemical characterization of bovine neurophysin - neurohypophyseal hormone complexes

As part of a continuing study of the neurophysin-peptide hormone system, the tripeptide analogue of vasopressin, L-methionyl-L-tyrosyl-L-phenylalaninamide, has been modified for use in photoaffinity labeling studies of the hormone binding site on neurophysin. The photo-sensitive peptide, L-methionyl-L-tyrosyl-p-azido-L-phenylalaninamide, was found by equilibrium dialysis in the absence of light to be reversibly bound to bovine neurophysin II. The dissociation constant, stoichiometry, and pH-dependence indicate that the interaction is similar to the interaction of hormone with neurophysin. The affinity reagent also competitively inhibits binding of L-methionyl-L-tyrosyl-L-phenylalaninamide which has been shown to competitively inhibit binding of hormone by neurophysin.

Exposing the noncovalent reagent-neurophysin complex to a high energy light source leads to formation of a covalent complex in moderate yields. Conditions for a better yield of covalent complex are being investigated. The covalent complex will then be fragmented, subjected to peptide mapping and sequencing in an attempt to determine which residues of the protein are found near or in the binding site.

Comparative studies using proteins from other species, including the human neurophysins isolated in an earler part of this project, should yield much information about the role of protein residues in the protein-peptide hormone interaction for these related species. (McCormick and Chaiken)

J. Investigations of protein conformation and function by semisynthesis

In a continuing program to study protein conformation and function by semisynthesis, investigations were made into the application of X-ray crystallogra-

phic techniques and some other methods to characterize semisynthetic ribonuclease-S' sequence variants. Several deletion and substitution analogues already prepared in this laboratory were successfully crystallized, including the species [des 16-20], [Gly 6, des 16-20], [p-F-Phe 8, des 16-20], (Gly 9, des 16-20], and [4-F-His 12, des 16-20]. These analogues, all derived by combining the appropriate synthetically synthesized [des 16-20] S-peptide with native S-protein, yielded crystals which by microscopic examination and preliminary X-ray diffraction studies were shown to be indistinguishable from Y-type crystals of fully native ribonuclease-S. This is indicative of conformational similarity in these variably perturbed species in the crystalline state.

In other aspects of study of 4-F-His 12-containing ribonuclease, preliminary proton nuclea magnetic resonance spectroscopic analyses were made which suggest that there is substantially more pH-induced conformational mobility in the [4-f-His 12, des 16-20] variant than in the native protein. Also, purification of S-protein and the [4-F-His 12, des 16-20] semisynthetic ribonuclease-S' by ribonuclease-specific affinity chromatography have yielded preparations which are totally inactive in both steps I and II of the enzymic mechanism of ribonuclease, supporting the view of active participation of His 12 in catalysis. The affinity chromatography approach also is being used to determine the peptide-protein association of the 4-F-His 12 analogue. (Chaiken, Ammon and Taylor)

K. Development and use of quantitative affinity chromatography to study interacting macromolecular systems

This project is a part of a continuing program to use affinity chromatography to determine quantitative parameters for macromolecule-involved interactions. For one model used, ribonuclease and a specific affinity matrix, competitive elutions with soluble ligand were used to measure equilibrium binding constants for ribonuclease and active site ligands. A detailed study was made of the behavior for the inactive semisynthetec ribonuclease-S' variant containing 4-F-Histidine at position 12. This inactive species was shown to bind substrates and inhibitors with a specificity related to that for native ribonuclease. The substrate binding property and quantitative aspects of inhibitor binding emphasize the crucial participation of His 12 in providing catalytically productive substrate binding and catalysis in native ribonuclease.

The ribonuclease affinity chromatography system also was used to investigate the possibility of measuring rate constants for protein-immobilized ligand interactions. The results so far indicate that the rate processes can be measured but that the calculated rate constants are remarkably smaller than those for protein and soluble ligand.

A project also has been initiated into the use of quantitative affinity chromatography to study multivalently interacting proteins, using a series of phosphorylcholine-specific myeloma IgA proteins as objects for study. (Taylor, Chaiken, Long and Eilat)

II. Studies on proteins and nucleic acids employing interaction with antibodies

A. Functional properties of immune response gene regulation of antibody response to nuclease

The ability to raise an immune response to specific antigens has recently been

shown to be under genetic control, linked to the major histocompatibility complex (MHC) in several species studied. In the mouse, a number of synthetic antigens have been found subject to this H-2 linked control, but only a few natural globular proteins have been studied as antigens, among these, staphylococcal nuclease. We have now tried to delineate the individual antigenic determinants on this protein which are responsible for this genetic control of the immune response in inbred strains of mice, in order to better understand the mechanisms involved. We have found that a single peptide fragment of the protein appears to be under the same control as the whole molecule. Furthermore, when antibodies made to the whole nuclease molecule are studied for their specificity, we have found that the H-2 linked immune response genes can control the response to individual determinants on the same molecule independently of one another, and therefore may be involved in the selection of specific B lymphocytes rather than only T cells. (Schechter)

B. Preparation and characterization of antibodies against neurophysin and other proteins and their use for conformational and biosynthetic studies

Purified antibodies specific for bovine neurophysins I and II have been obtained from rabbit antisera against the proteins conjugated to polyalanylpolylysine, with final purifications of these antibodies by affinity chromatography on immobilized neurophysin matrices. The high level of immunological cross-reactivity of neurophysins I and II was verified by the affinity chromatographic technique, indicating the extensive structural similarity between these species. Affinity chromatographic separations of specific antibodies were followed using [$^{14}$C-carbamoyl)neurophysin II in two radioimmunoassays developed during this study.

Purified neurophysin antibodies were bound to Sepharose to generate an immunoadsorption matrix. This matrix is capable of binding not only native neurophysin but also a protein derivative in which the hormone binding site is blocked. This matrix will be used mainly to try to isolate a postulated neurophysin precursor from hypothalamic tissue or other appropriate source. Preliminary results indicate the presence in hypothalamic extracts of species, which bind to the immunoadsorbant, of molecular weight higher than that of neurophysin.

A program has been initiated to prepare antibodies against the thermophilic protease, thermolysin, as well as some large fragments of this protein. This study is designed as an approach to understanding the conformational propensities of isolated protein domains. Separation of ribonuclease antibodies specific for particular regions of the protein also is currently in progress. (Chaiken, McCormick and Fontana)

C. Radioimmunoassay of creatine kinase: a model for radioimmunoassay of human serum enzymes

Human serum enzyme assays for clinical diagnosis usually make use of measurements of catalytic activity. In many situations, this may not be ideal; e.g. isoenzymes reflecting different damaged tissues may be indistinguishable or inactive enzymes may be released. Radioimmunoassay offers a very sensitive and specific way in which to measure enzyme concentration and to distinguish iso-

enzymes. Creatine kinase was chosen as a model for this approach because of its clinical importance and its simple isoenzyme distribution. The enzymes from human skeletal muscle (M) and brain (B) have been purified, used as immunogens to raise antibodies, and labeled with $^{125}I$. Radioimmunoassays for each of these enzymes were developed and were found to correlate well with standard enzyme assays in clinical use. An attempt is now being made to develop a specific assay for the hybrid MB species. (Schechter)

D. Interaction of complement proteins with leukocytes, erythrocytes, and antibodies

The ability of phagocytic and lymphoid cells, including the macrophage, polymorphonuclear leukocyte (PMN), and B lymphocyte, to recognize and bind to immune complexes (IC) has been attributed to the interaction between an IC bound form of the third component of complement (C3) and cell surface receptors specific for this protein. We are attempting to measure directly the equilibrium and kinetic parameters of the binding between activation products of human C3 and their receptors on human blood cells. The activation fragments C3b, C3c, and C3d have been generated by digestion with porcine elastase and subsequently purified. Each fragment has been radiolabeled by reductive methylation using formaldehyde and ($^{3}H$) sodium borohydride. The interaction with erythrocytes and different leukocyte populations will be assessed using a direct binding assay where equilibrium saturation, time course, and competition experiments are expected to characterize the physio-chemical nature of these interactions. In addition, specific antibodies have been raised in sheep and rabbits to each of these fragments and will be used for (a) probes to identify receptor binding sites in C3b and (b) developing specific radioimmunoassays to assess *in vivo* complement activation. (Tack, Lorenz and Schechter)

E. Immunochemical studies of RNA

We have studied single stranded (ss) RNA binding immunoglobulins in the sera of patients with systemic lupus erythematosus as part of a project to obtain antibodies with conformational specificity for nucleic acids. The antibody population that bound polyribonucleotides and the population that bound polydeoxyribonucleotides appear to be distinct. The anti ss-RNA antibodies preferentially bound native ssRNA (as compared with synthetic polyribonucletides) with an increasing affinity for the higher molecular weight RNA's. We have also developed an assay for detection of specific RNA antibodies produced *in vitro* by spleen cells from old female NZB/NZW F1 mice. This assay enables one to test the effect of different therapeutic agents at the immediate site of antibody synthesis. (Eilat and Schechter)

III. Properties of nucleic acids

A. Nucleic acid chromatography and chemical modification

The ethidium cation of the salt ethidium bromide has been previously shown by others to be a sequence specific nucleic acid intercalating agent. We have demonstrated the interaction of gel bound ethidium ion into nucleic acid double helices by a variety of biophysical techniques including fluorescence. Preliminary experiments indicate that crude transfer ribonucleic acid can be fractio

ated to some degree on this column using elution with a linear sodium chloride gradient. It has also been found that $tRNA^{Phe}_{E.\ coli}$ can be selectively reacted at the X-base with N-hydroxysuccinimide esters of fluorescent compounds. This reaction has been studied as a source of information about the function of this group and as a spectroscropic probe of tRNA structure. (Thomas and Schechter)

## IV. Studies on protein components of P. aurelia

### A. Purification and examination of the substructure of i-antigen from P. aurelia

The immobilization antigen (i-antigen) of Paramecium is a surface protein which is distributed over 90 percent of the cell membrane. When cells of a particular antigenic type are placed in homologous antisera, they become immobilized and eventually die. While a clonal population of cells generally expresses only one serotype at any given time, cells are capable of expressing as many as twelve different antigens. Serotype expression, as well as the phenomenon of mutual exclusion, is under the control of a complex interaction of genes cytoplasm and cellular environment.

The antigen system in paramecium has received considerable interest as a possible model for intra-cellular differentiation.

Recent controversy has arisen over the precise substructure of certain of the i-antigens. Descrepancies in various physical and chemical measurements have arisen because methods used to purify the i-antigen have failed to remove trace amounts of contaminating proteins including some with limited proteolytic activity. Further characterization of the structure of the i-antigens requires a reexamination of the methods of purification.

We have developed a method of purification for antigens A and D by affinity chromatography using specific antibodies attached to Sepharose. Subsequent studies on the substructure of these antigens revealed limited proteolytic digestion of the antigens during their purification. Addition of iodoactamide to the extraction and purification buffers inhibited any detectable degradation. The product of this purification appears to consist of subunits as evidenced by reduction and alkylation in 8M urea.

In addition, fractionation of the product derived from "pure" clones of serotype A and D revealed the presence of "secondary" i-antigens which are not detected in immobilizing tests on live cells. (Steers and Lee)

### B. Isolation and characterization of the mating substances (M-agglutinins)

Mating (or conjugation) in Paramecium occurs between complementary cells during special periods of reactivity. The interaction between reactive cells shows a high degree of specificity. When cells of opposite mating type are mixed under the appropriate conditions, they agglutinate into clumps containing from two to twenty animals. The agglutination phase is followed by a separation of the clumped cells into complementary pairs. Following a reciprocal exchange of gametic nuclei, the cells separate and undergo mitotic division.

The agglutinating reaction of paramecium, which triggers the cellular events leading to nuclear exchange and cell division, is reminiscent of another class of agglutinins - the lectins. The project outlined here is

directed toward the isolation and characterization of the mating substances (M-agglutinin) of paramecium. Such characterization should lead to an understanding of the molecular basis for the specificity of agglutination in the mating reaction as a model for describing this specific cell-cell interaction. (Steers, Barnett and Lee)

C. Purification and characterization of the myoglobins from P. tetramer

The protozoan Paramecium aurelia has been shown to contain a respiratory pigment which appears homologous to mammalian myoblobin. This pigment is present in five electrophoretically different forms with isoelectric points of 5.54, 5.18, 4.98, 4.51 and 4.16 respectively. Purification of these pigments involves ammonium sulfate fractionation, gel filtration through Sephadex G-75 and G-50 and electrophoresis employing either polyacrylamide gels or isoelectric focusing. The molecular weights of the five pigments is approximately 16,000. The physiological role of these pigments remains obscure at this time. Whether the electrophoretic forms show functional heterogeneity or are functionally homogeneous is also not known at this time. Work on this project is in abeyance at the present time. It will be resumed in the near future. (Steers)

V. Human interferon

Since the amount of interferon in biological fluids is very low, one goal was to produce human lymphoblastoid interferon in large quantities. The cell line Namalva was induced with Newcastle disease virus, strain B1. The growth of Namalva cells to volumes of 200 liters was accomplished using the pilot plant facilities at the NIH (NIAMDD) Bldg. 6 under the supervision of Mr. David Rogerson. Subsequently interferon was produced by dilution of virus induced cultures with serum-free medium to final volumes of up to 800 liters. These preparations have yielded approximately 5-10 mg lymphoblastoid interferon. We are also engaged in the large scale production of lymphoblastoid interferon in conjunction with the Frederick Cancer Research Center.

Studies examining the growth of Namalva cells in serum-free medium are also in progress. It was found that these cells were capable of growing in at least one serum-free medium although optimum growth was not obtained. Attempts are being made to increase the cell densities and growth rate of these cultures by the addition of a number of hormones and small proteins known to stimulate cell growth.

Methods for the purification of lymphoblastoid interferon from the crude tissue culture fluids include trichloroacetic acid precipitation, immunoabsorbant affinity chromatography, ion-exchange chromatography, isoelectric focusing and sodium dodecyl sulfate polyacrylamide gel electrophoresis. Using several combinations of these procedures, lymphoblastoid interferon has been partially purified to at least $1\text{-}2 \times 10^7$ units/mg protein. Experiments are now in progress to use all of the above methods in an attempt to achieve a homogeneous preparation of lymphoblastoid interferon. (Anfinsen, Bose, Bridgen, Corley, Ruegg, Smith, Zoon)

| SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space) | U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT | PROJECT NUMBER Z01 AM 25,000-04 LCB |
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PERIOD COVERED
July 1, 1976 to September 30, 1977

TITLE OF PROJECT (80 characters or less)

Isolation, Structural Determination, and Synthesis of Human Interferon

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

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| | P. J. Bridgen | Visiting Fellow | LCB NIAMDD |
| | L. Corley | Chemist | LCB NIAMDD |
| | U. Th. Ruegg | Visiting Fellow | LCB NIAMDD |
| | M. Smith | Guest Worker | LCB NIAMDD |
| | K. Zoon | NIAID Fellow | LCB NIAMDD |

COOPERATING UNITS (if any)

LAB/BRANCH
Laboratory of Chemical Biology

SECTION
Protein Chemistry Section

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20014

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☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Since the amount of interferon in biological fluids is very low, one goal was to produce human lymphoblastoid interferon in large quantities. The cell line Namalva was induced with Newcastle disease virus, strain B1. The growth of Namalva cells to volumes of 200 liters was accomplished using the pilot plant facilities (NIAMDD) Bldg. 6 under the supervision of Mr. David Rogerson. Subsequently interferon was produced by dilution of virus induced cultures with serum-free medium to final volumes of up to 800 liters. These preparations have yielded approximately 5-10 mg lymphoblastoid interferon. Studies examining the growth of Namalva cells in serum-free medium are also in progress. It was found that these cells were capable of growing in at least one serum-free medium although optimum growth was not obtained. Methods for the purification of lymphoblastoid interferon from the crude tissue culture fluids include trichloroacetic acid precipitation, immunoabsorbant-affinity chromatography, ion-exchange chromatography, isoelectric focusing and sodium dodecyl sulfate polyacrylamide gel electrophoresis. Lymphoblastoid interferon has been partially purified to at least 1-2 x $10^7$ units/mg. Experiments are now in progress to use all of these methods to achieve a homogeneous preparation of interferon.

Project Description:

Growth of Namalva Cells and Production of Lymphoblastoid Interferon. Namalva cells were grown at 37°C in RPMI 1640 supplemented with 25 mM HEPES and 10% heat inactivated (56°C/30 min) fetal calf serum in either 17 liter carboyx (10 liters of culture) or in a 100 gallon stainless steel fermenter (200 liters of culture). At saturation density (1.6-2.5 x $10^6$ cells/ml) the cultures were induced with Newcastle disease virus, strain B1 (25 hemagglutination units/$10^6$ cells) and after 1 hr were diluted to approximately 5 x $10^5$ cells/ml with serum-free medium (RPMI 1640). The culture was then incubated an additional 26-39 hr at 35°C. Using this dilution procedure for the production of lymphoblastoid interferon the interferon yield per cell was increased 2-4 fold over that of saturation density cultures. The production costs were reduced, and furthermore, the purification was aided by increasing the biological specific activity of the lymphoblastoid interferon in the crude tissue culture fluid. The interferon containing culture fluids were harvested by centrifugation using De Laval centrifuges Models Gyrotest and 108. Maximum yields of 2-4 x $10^9$ units per culture were obtained using this procedure. Namalva cells were also grown in serum-free medium (RPMI 1640) supplemented with methyl cellulose and bovine serum albumin. We are now examining interferon production in these cultures.

Concentration of Namalva Interferon by Precipitation. The lymphoblastoid interferon present in the crude tissue culture fluid was concentrated by precipitation with trichloracetic acid (5% w/v final concentration). Although no purification was obtained, the interferon was concentrated 150 fold. In addition, the trichloroacetic acid (TCA) inactivates the Newcastle disease virus and thus permits the immediate processing of the crude interferon preparation. The TCA was removed by gel filtration using Sephadex G-25 medium equilibrated with phosphate-buffered saline pH 7.4 and .02% sodium azide.

Purification of Lymphoblastoid Interferon. Lymphoblastoid interferon was purified an average of 293 fold by anti-leukocyte interferon affinity chromatography. The average recovery of the interferon was 80%. The antibody was prepared in sheep using as an antigen, material that had been partially purified by gel filtration through a Sephadex G-100 column. Anti-interferon in the $\gamma$-globulin fraction was purified before attachment to Sepharose 4B by passage through an affinity column bearing the protein impurities that might be present in crude interferon-containing culture fluid (serum proteins and proteins of the buffy coat cells, allantoic fluid, and Sendai virus). The results are summarized in our work on the partial purification of interferon in Proc. Nat. Acad. Sci. 71, 3139, 1974. Similarly, we are now preparing antiserum to lymphoblastoid interferon which was partially purified by anti-leukocyte interferon affinity chromatography. The interferon was further purified 6-10 fold by SP-Sephadex ion exchange chromatography. The interferon will be further purified by isoelectric focusing and preparative sodium dodecyl sulfate polyacrylamide slab gel electrophoresis. We have shown that lymphoblastoid interferon can be completely recovered from an SDS gel both with and without staining with Coomassie brilliant blue R250. This is a powerful tool

which permits us to not only recover all of the interferon but also enables us to correlate a specific stained protein band with a band of interferon activity.

Sequencing of lymphoblastoid interferon. Following the purification of lymphoblastoid interferon, the isolated minute amounts will be subjected to sequence analysis on a solid phase sequenator using $^{35}S$ phenyl isothiocyanate (PITC). The phenylthiohydantoin will be identified by thin layer chromatography on polyamide plates followed by autoradiography or high pressure liquid chromatography. To determine the complete sequence of the interferon molecule, the purified labeled interferon will be fragmented two different ways to produce overlapping peptides using: 1) trypsinization of succinylated interferon which produces COOH-terminal arginine peptides; and 2) cyanogen bromide treatment of the purified interferon, which results in the formation of COOH-terminal homoserine lactone peptides. These peptides will then be separated by chromatography and sequenced on a solid phase sequencer. By comparing the amino acid composition of the two sets of overlapping peptide fragments, the correct sequence can be obtained.

Once the sequence has been determined, the chemical synthesis of the polypeptide portion of the interferon molecule will be attempted using a combination of the Merrifield solid-phase technique and classical fragment synthesis methods.

The carbohydrate components of fibroblast interferon have been studied using radioactive glucosamine incorporation to label the sugar moieties. Digestion of such radioactive interferon with a broad spectrum of glycosidases (an enzyme-mixture consisting of neuraminidase, β-galactosidase, α-mannosidase, N-acetyl glucosaminidase, etc.) removes 85-90% of the labelled sugar without loss of antiviral activity. At the same time the interferon loses much of its heterogeneity, presumably due to variable contents of sialic acid. Dorner et al. have shown that the terminal sequence of the carbohydrate moiety attached to the polypeptide chain of interferon is sialic acid-galactose and yields one fairly sharp zone upon electrofocusing. On treatment with glycosidases, the molecular weight of leukocyte interferon was reduced by about $4 \times 10^3$. (This part of our work on interferon has been summarized in J. Biol. Chem. 251, 1659, 1976.)

Since our recent work on interferon shows that the antiviral activity of interferon does not depend on the carbohydrate moiety attached to its polypeptide chain, there is a great possibility that the chemically synthesized interferon will exhibit antiviral activity even without having its carbohydrate moiety.

The large scale production, purification and sequencing of interferon will be continued and if successful, studies will be initiated to synthesize it and determine the antiviral activity and the rate of catabolism of this molecule compared to the control purified interferon.

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PERIOD COVERED

July 1, 1976 to September 30, 1977

TITLE OF PROJECT (80 characters or less)

Purification and Examination of the Substructure of i-Antigen from P. aurelia.

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

| | | | |
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| PI: | Edward Steers, Jr. | Research Biologist | LCB NIAMDD |
| OTHER: | Clifford E. Lee | Physical Science Technician | LCB NIAMDD |

COOPERATING UNITS (if any)

LAB/BRANCH

Laboratory of Chemical Biology

SECTION

Protein Chemistry Section

INSTITUTE AND LOCATION

NIAMDD:NIH; Bethesda, Md. 20014

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The immobilization antigen (i-antigen of Paramecium is a surface protein which is distributed over 90 percent of the cell membrane. While a clonal population of cells generally expresses only one serotype at any given time, individual cells are capable of expressing as many as twelve different antigens. Serotype expression, as well as the phenomenon of mutual exclusion, is under the control of a complex interaction of genes, cytoplasm and cellular environment. Discrepancies in various physical and chemical measurements have arisen because methods used to purify the i-antigen have failed to remove trace amounts of contaminating proteins including some with limited proteolytic activity. We have developed a method of purification for i-antigens A and D using immunoaffinity chromatography. The product of this purification appears to consist of unequal subunits following reduction and alkylation.

In addition, fractionation of the product derived from "pure" clones of serotpye A and D revealed the presence of "secondary i-antigens which are not detected in immobilizing tests on live cells.

Objectives: To purify and characterize the immobilizing (i-antigens) from P. tetraurelia.

Methods Employed: Purification on anti-i-antigen sepharose 4B (affinity chromatography). Purification was monitored by polyacrylamide gel electrophoresis (standard, SDS, and urea) and by immunodiffusion in agar gels. Characterization- reduction and alkylation in 8M urea. The product was examined by gel electrophoresis, ultracentrifugation, isoelectric focusing, amino acid analysis and immunodiffusion.

Major Findings: The i-antigen purified by previously published procedures is not pure. Small amounts of contaminating protein contained a protease which was markedly stimulated by reducing concentrations of 2-mercaptoethanol or dithiothreitol. Earlier work in our laboratory showed that this proteolytic activity digested the i-antigen, and other test proteins, into fragments averaging 20,000 to 30,000 molecular weight. The degradation occurred during the reduction of the protein in either 8M urea or 0.1% SDS. Alternate methods of purification were necessary to obtain a homogenous product free of proteolytic activity. Ion-exchange chromatography resulted in a fraction which was homogeneous and free of degradative activity. This method proved to be impractical, however, as a preparative procedure. Losses of 50% of the applied sample occurred and selective cuts of the desired fractions resulted in further losses. An alternate method of affinity chromatography was developed using the ion-exchange purified antigen to induce specific antibody.

The substructure of the i-antigen was examined by reducing the protein with DTT in 8M urea followed by alkylation with iodoacetamide. Polyacrylamide electrophoresis on SDS containing gels showed a heterogeneous product which contained approximately fifteen fractions ranging in molecular weight from 35,000 to 270,000 (native molecular weight, 310,000). Parallel samples of i-antigen which had been purified by earlier procedures resulted in "subunits" of 25,000 to 40,000 molecular weight. These results suggested that while the proteolytic activity had been removed by affinity chromatography, limited proteolysis occurred during the extraction stages. The question of possible subunit structure remained undefined. It became necessary to add iodoacetamide (20mg/ml) to the extraction buffers to inhibit the degradative activity. i-antigen purified by this procedure showed no alkylated cysteine on amino acid analysis and was immunologically identical to the antigen purified by the standard procedure. Antigen purified in iodoacetamide-containing buffers showed no proteolytic activity when assayed at each stage of the purification, however, i-antigen purified under these conditions still appeared heterogeneous on polyacrylamide SDS gels. A main component of 200,000 to 220,000 molecular weight was found with several smaller components. The reduced and alkylated product lacked any cross reactivity with antisera against native antigen, but antisera against the reduced - alkylated product showed cross-reactivity to the native i-antigen.

An additional observation resulting from these studies was the finding, in certain clonal populations of "pure" serotypes, of significant amounts of non-cross reacting i-antigen which were undetectable in immobilization tests. These "secondary" i-antigens appear to be either cytoplasmically located or, if on the surface of the cells, sterically blocked from reacting with homologous antisera and therefore undetectable by the usual immobilization test.

The presence of such secondary i-antigens require a reexamination of the theories of mutual exclusion and the mechanisms controlling antigen expression.

Significance to Biomedical Research: Although researchers agree that the protein responsible for the immobilizing reaction in paramecium is a large, fibrous protein of approximately 310,000 molecular weight, there is conflicting evidence concerning the existence and number of subnunits. Resolution of this question will give insight into the genetic control of i-antigen structure and aid in understanding the cross-reactions previously observed in gel-diffusion tests. The detection of "secondary" antigens in stable clonal populations of cells requires a reexamination of the concepts of mutual exclusion in this organism.

Proposed Course: Experiments are currently underway to characterize antigens A and D from strain 51.7s of P.tetraurelia. This characterization has been seriously hampered by an inability to isolate a homogeneous product which was not modified by a contaminating protease. In addition, experiments are being designed to localize "secondary" antigens that have been identified in clonal populations that were previously believed to be pure for a given serotype.

Publication:

Steers, E. and Davis, Jr. R.H.: A Reexamination of the Immobilization Antigen from P. aurelia. Comparative Biochem. & Physiol. 56B, 195-197, 1977.

Davis, Jr. R.H. and Steers, Jr. E. Purification of the i-Antigen from P.tetraurelia by Immunoaffinity Chromatography. In Press. 1977.

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PERIOD COVERED

July 1, 1976 to September 30, 1977

TITLE OF PROJECT (80 characters or less)

Purification and Characterization of the Myoglobins from P. tetraurelia.

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: Edward Steers, Jr. Research Biologist LCB NIAMDD

COOPERATING UNITS (if any)

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The protozoan Paramecium aurelia has been shown to contain a respiratory pigment which appears homologous to mammalian myoblobin. This pigment is present in five electrophoretically different forms with isoelectric points of 5.54, 5.18, 4.98, 4.51 and 4.16 respectively. Purification of these pigments involves ammonium sulfate fractionation, gel filtration through Sephadex G-75 and G-50 and electrophoresis employing either polyacrylamide gels or isoelectric focusing. The molecular weights of the five pigments is approximately 16,000. The physiological role of these pigments remains obscure at this time. Whether the electrophoretic forms show functional heterogeneity or are functionally homogeneous is also not known at this time.



FS-6040

Work on this project is in abeyance at the present time. It will be resumed in the near future.

| SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space) | U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT | PROJECT NUMBER Z01 AM 25,003-07 LCB |
|---|---|---|

PERIOD COVERED
July 1, 1976 to September 30, 1977

TITLE OF PROJECT (80 characters or less)

Investigations of Protein Conformation and Function by Semisynthesis

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

| | | | |
|---|---|---|---|
| PI: | I.M. Chaiken | Research Chemist | LCB NIAMDD |
| | H.L. Ammon | Visiting Scientist | LCB NIAMDD |
| | H.C. Taylor | Chemist | LCB NIAMDD |

COOPERATING UNITS (if any)

LAB/BRANCH
Laboratory of Chemical Biology

SECTION
Protein Chemistry Section

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20014

| TOTAL MANYEARS: | PROFESSIONAL: | OTHER: |
|---|---|---|
| 1.6 | 1.3 | 0.3 |

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

In a continuing program to study protein conformation and function by semisynthesis, investigations were made into the application of X-ray crystallographic techniques and some other methods to characterize semisynthetic ribonuclease-S' sequence variants. Several deletion and substitution analogues already prepared in this laboratory were successfully crystallized, including the species [des 16-20], [Gly 6, des 16-20], [p-F-Phe 8, des 16-20], (Gly 9, des 16-20], and [4-F-His 12, des 16-20]. Microscopic examination and preliminary X-ray diffraction studies indicate conformational similarity between these variably perturbed species, and fully native complex, in the crystalline state. Other aspects of study of 4-F-His 12-containing ribonuclease include proton nuclear magnetic resonance spectroscopic analyses (showing substantially more pH-induced conformational mobility in [4-F-His 12, des 16-20] variant than in native protein); and purification of S-protein and [4-F-His 12, des 16-20] semisynthetic ribonuclease-S' by ribonuclease-specific affinity chromatography, reaffirming the view of active participation of His 12 in catalysis. Affinity chromatography also is being used to determine peptide-protein association of the 4-F-His 12 analogue.

Project Description:

Objectives:

1) To prepare and purify semisynthetic ribonuclease-S' sequence variants with particular amino acid substitutions and deletions in the S-peptide region (corresponding to residues 1 through 20 of native ribonuclease).

2) To study conformational properties of these analogues by X-ray crystallographic methods.

3) To study both catalytic properties and S peptide-S protein association with affinity chromatographic methodology.

Methods Employed: The semisynthetic ribonuclease-S' analogues were prepared by combining S peptide analogues made chemically by solid phase peptide synthesis with native S-protein obtained by enzymic cleavage of native bovine pancreatic ribonuclease. The [4-F-His 12, des 16-20] ribonuclease-S analogue and S-protein were extensively purified by affinity chromatography on Sepharose-5'-(4-aminophenylphosphoryl)-uridine-2'(3')phosphate. The microenvironments of the histidine regions of the 4-F-His 12 complex and native ribonuclease-S were studied with 220 MHz fourier transform protein nuclear magnetic resonance. X-ray diffraction techniques were employed to verify the overall conformational similarity of semisynthetic and native ribonuclease-S species.

Major Findings: Crystals of the [des 16-20], [Gly 6, des 16-20], [p-F-Phe 8, des 16-20], [Gly 9, des 16-20] and [4-F-His 12, des 16-20] ribonuclease S' analogues were grown at pH 5.3 and shown by visual examination and preliminary X-ray diffraction experiments to be identical to Y-form crystals of native ribonuclease-S. The finding suggests the absence of any gross conformational differences between the semisynthetic and native enzymes. Both S-protein and the [4-F-His 12, des 16-20] analogue, following affinity column purification, were found to be without enzymic activity. These results indicate the need for a ribonuclease-like conformation and for a histidine of appropriate pKa at position 12 for activity. The ribonuclease affinity column is being used to investigate the dissociation of the 4-F-His 12 analogue, revealing preliminarily a fairly typical ribonuclease-S'-like value for $K_d$ of $10^{-7}$ M.

Significance to Biomedical Research: The general results of the semisynthesis program are designed to permit an increasingly complete understanding of the molecular interactions which govern protein conformation and function. The recent X-ray crystallographic results specifically described here create the possibility for full scale engineering of ribonuclease-like sequence variants to test many current ideas on these molecular interactions.

Proposed Course of Action:

1) Determination of the complete three-dimensional structures of several of the analogues with X-ray crystallographic techniques.

2) Preparation and X-ray diffraction analysis of the complex of [4-F-His 12, des 16-20] semisynthetic ribonuclease-S' with UpA, a typical ribonuclease substrate.

3) Design of analogues of semisynthetic ribonuclease-S' to further define the involvement of particular types of amino acid information in the various properties of the ribonuclease system.

Publications:

Pandin, M., Padlan, E.A., DiBello, C., and Chaiken, I.M.: Crystallization of Solid Phase-derived Semisynthetic Ribonuclease-S'. in Peptides 1976 - Proc. 14th Euro. Peptide Symp., A. Loffet, ed. (Presses Universitaires de Bruxelles): 319, 1976.

Chaiken, I.M., Taylor, H.C., and Ammon, H.L.: Crystal Properties of [des 16-20] Semisynthetic Sequence Variants of Ribonuclease-S'. J. Biol. Chem., in press.

| SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space) | U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT** | PROJECT NUMBER ZO1 AM 25,004-04 LCB |
|---|---|---|

PERIOD COVERED

July 1, 1976 to September 30, 1977

TITLE OF PROJECT (80 characters or less)

Quantitative Affinity Chromatography to Study Interacting Macromolecular Systems

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

| | | | |
|---|---|---|---|
| PI: | H.C. Taylor | Chemist | LCB NIAMDD |
| | I.M. Chaiken | Research Chemist | LCB NIAMDD |
| | R. Long | Guest Worker | LCB NIAMDD |
| OTHER: | D. Eilat | Visiting Fellow | LCB NIAMDD |

COOPERATING UNITS (if any)

LAB/BRANCH

Laboratory of Chemical Biology

SECTION

Protein Chemistry Section

INSTITUTE AND LOCATION

NIAMDD:NIH; Bethesda, Md. 20014

| TOTAL MANYEARS: | PROFESSIONAL: | OTHER: |
|---|---|---|
| 1.4 | .3 | 1.1 |

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

This project is a part of a continuing program to use affinity chromatography to determine quantitative parameters for macromolecule-involved interactions. For one model used, ribonuclease and a specific affinity matrix, competitive elutions with soluble ligand were used to measure equilibrium binding constants for ribonuclease and active site ligands. A detailed study was made of the behaviour for the inactive semisynthetec ribonuclease-S' variant containing 4-F-histidine at position 12. This inactive species was shown to bind substrates and inhibitors with a specificity related to that for native ribonuclease. The substrate binding property and quantitative aspects of inhibitor binding can be correlated with the participation of His 12 in substrate binding and catalysis in native ribonuclease.

The ribonuclease affinity chromatography system also was used to investigate the possibility of measuring rate constants for protein - immobilized ligand interactions.

A project also has been initiated into the use of quantitative affinity chromatography to study multivalently interacting proteins, using a series of phosohorylcholine - specific myeloma IgA proteins as objects for study.

Project Description:

Objectives:

1) To apply previously developed technology in quantitative affinity chromatography of ribonuclease to the study of equilibrium properties of ligand binding of specific semisynthetic ribonuclease-S' sequence variants.

2) To develop further the general quantitative use of affinity chromatography to measure rate constants for ligand-protein interactions.

3) To investigate the use of quantitative affinity chromatography to study ligand binding properties of multivalent proteins.

Methods Employed: Sepharose-5'-(4-aminophenylphosphoryl) uridine-2' (3') -phosphate was used as the ribonuclease-specific affinity matrix. Competitive elution analyses with a series of active site ligands were carried out using a [4-F-His 12, des 16-20] semisynthetic ribonuclease-S' species prepared with tritium - labelled Phe 8. Elution of zones of radioisotope - labelled protein were followed by scintillation counting, with results of sets of elutions with a particular soluble ligand analyzed using a previously derived mathematical model. For determination of rate constants, selected competitive elutions of both tritiated, normal-sequence semisynthetic ribonuclease-S' and [$^{14}$C-carbamoyl] ribonuclease-A were carried out on the ribonuclease affinity matrix, either with no soluble ligand or with saturating soluble ligand.

Phosphorylcholine - Sepharose was prepared and used for competitive elution analyses (by soluble phosphorylcholine) of phosphorylcholine-specific myeloma IgA (as TEPC-15). The latter was radioisotopically labelled and converted to bivalent IgA monomer.

Major Findings: Chromatographic analyses of tritiated [4-F-His 12,des 16-20] semisynthetic ribonuclease-S' by competitive elution showed that this inactive species still had an active site able to recognize and bind significantly to ribonuclease substrates as well as ribonuclease nucleotide inhibitors. The results increase our understanding of the normal role of His 12 in native ribonuclease substrate binding and catalysis. The successful results with 4-F-His 12 complex also demonstrate the general utility of the quantitative affinity chromatographic approach to obtain otherwise difficult-to-measure data for inactive noncovalent complex - ligand interactions.

The attempts to obtain rate constants for active site ligand - ribonuclease interactions has indicated so far that binding of protein to immobilized ligand is significantly altered from the free solution binding but that obtaining at least relative rate constants is possible by the chromatographic approach.

Preliminary data using myeloma IgA bivalent monomer indicate that the chromatographic approach can allow study of polyvalent as well as monovalent protein - ligand interactions.

Significance to Biomedical Research: In general, the increasing development of quantitative affinity chromatography will be of importance in allowing the elucidation of many kinds of interacting processes involving not only proteins but other macromolecular or even cellular systems. The approach should provide an alternative to several imprecise or impractical types of techniques

now used to study complex, biologically important systems.

Proposed Course of Project:

1) Further exploration of rate constant measurements with the ribonuclease system.

2) Completion of the characterization of competitive elution properties for specific myeloma IgA species on phosphorylcholine - Sepharose and development of a mathematical model to describe behavior for these bivalent systems.

3) Exploration of use of quantitative affinity chromatography to study more complex biologically important interactions.

Publications:

Taylor, H.C., and Chaiken, I.M.: Active site ligand binding by an inactive ribonuclease-S analogue: A quantitative affinity chromatographic study. J. Biol. Chem. in press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)

U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE
PUBLIC HEALTH SERVICE
NOTICE OF
INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 AM 25,006-04 LCB

PERIOD COVERED

July 1, 1976 to September 30, 1977

TITLE OF PROJECT (80 characters or less)

Characterization of Bovine Neurophysin - neurohypophyseal Hormone Complexes

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT


| | | | |
|---|---|---|---|
| PI: | W.M. McCormick | Chemist | LCB NIAMDD |
| OTHER: | I.M. Chaiken | Research Chemist | LCB NIAMDD |


COOPERATING UNITS (if any)

LAB/BRANCH

Laboratory of Chemical Biology

SECTION

Protein Chemistry Section

INSTITUTE AND LOCATION

NIAMDD:NIH; Bethesda, Md. 20014

| TOTAL MANYEARS: | PROFESSIONAL: | OTHER: |
|---|---|---|
| 1.1 | 0.2 | 0.9 |

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)


As part of a continuing study of the neurophysin-peptide hormone system, the tripeptide analogue of vasopressin, L-methionyl-L-tyrosyl-L-phenylalaninamide, has been modified for use in photoaffinity labeling studies of the hormone binding site on neurophysin. The photo-sensitive peptide, L-methionyl-L-tyrosyl-p-azido-L-phenylalaninamide, was found by equilibrium dialysis in the absence of light to be reversibly bound to bovine neurophysin II. The dissociation constant, stoichiometry, and pH-dependence indicate that the interaction is similar to the interaction of hormone with neurophysin. The affinity reagent also competitively inhibits binding of L-methionyl-L-tyrosyl-L-phenylalaninamide which has been shown to competitively inhibit binding of hormone by neurophysin.

Exposing the noncovalent reagent-neurophysin complex to a high energy light source leads to formation of a covalent complex in moderate yields. Conditions for a better yield of covalent complex are being investigated. The covalent complex will then be fragmented, subjected to peptide mapping and sequencing in an attempt to determine which residues of the protein are found near or in the binding site.

Project Description:

A major objective of this project is to study the interaction of hormone analogues with neurophysin in order to obtain information about the hormone binding site on the neurophysin molecule. The identification of amino acid residues of the protein involved in the peptide-protein binding interaction will tell much about the environment of the binding site and should yield important information about the 3-dimensional structure of the neurophysins.

A major technique being used is known as photoaffinity labeling. The photo label which has been designed for use in this study is L-methionyl-L-tyrosyl-p-azido-L-phenylalaninamide. This reagent has been found to bind to bovine neurophysin II reversibly and specifically in the dark, the manner of interaction being analogous to hormone binding by neurophysin. When photolyzed, some of the neurophysin becomes covalently labeled with affinity label. Techniques to be used in order to determine the location of the label on the protein include enzymic digestion, peptide mapping and sequencing.

Comparative labeling studies will be done with neurophysins from other species in order to identify similarities and differences in the binding sites. Also, other labels with the photoreactive group in different positions may increase the information obtained about the binding site.

Information about peptide-protein interactions is important for understanding many biochemical interactions. We hope to learn what arrangement of residues is necessary to bring about the specificity of the binding interaction. Experimental proof is now beginning to support the existance of a precursor from which the neurophysins and hormones may be derived. Trapping the neurophysin in the bound state may help to explain the significance of this precursor molecule. It will be interesting to determine if the labeled neurophysin retains a conformation similar to the unlabeled or whether occupying the binding site will alter conformation significantly and thus allow the expression of many of the properties of storage, transport and release that have been studied.

Publications:

Diliberto, M.J., Axelrod, J., and Chaiken, I.M.: Effects of ligands on enzymic carboxyl-methylation of Neurophysins. Biochem. Biophys. Res. Commun. 73: 1063, 1976.

| SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space) | U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT | PROJECT NUMBER Z01 AM 25,007-04 LCB |
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PERIOD COVERED
July 1, 1976 to September 30, 1977

TITLE OF PROJECT (80 characters or less)
Antibodies against neurophysin and other proteins and their use for conformational and biosynthetic studies

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

| | | | |
|---|---|---|---|
| P.I.: | I.M. Chaiken | Research Chemist | LCB NIAMDD |
| OTHER: | W. McCormick | Chemist | LCB NIAMDD |
| | A. Fontana | Visiting Scientist | LCB NIAMDD |

COOPERATING UNITS (if any)

LAB/BRANCH
Laboratory of Chemical Biology

SECTION
Protein Chemistry Section

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20014

| TOTAL MANYEARS: | PROFESSIONAL: | OTHER: |
|---|---|---|
| 0.4 | 0.3 | 0.1 |

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☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Purified antibodies specific for bovine neurophysins I and II have been obtained from rabbit antisera against the proteins conjugated to polyalanyl-polylysine, with final purifications of these antibodies by affinity chromatography on immobilized neurophysin matrices. The high level of immunological cross-reactivity of neurophysins I and II was verified by the affinity chromatographic technique, indicating the extensive structural similarity between these species. Purified neurophysin antibodies were bound to Sepharose to generate an immunoadsorption matrix. This matrix binds not only native neurophysin but also a protein derivative in which the hormone binding site is blocked. This matrix will be used mainly to try to isolate a postulated neurophysin precursor from hypothalamic tissue or other appropriate source. Preliminary results indicate the presence in hypothalamic extracts of species, which bind to the immunoadsorbant, of molecular weight higher than that of neurophysin.

A program has been initiated to prepare antibodies against thermolysin, as well as some large fragments of this protein. This study is designed as an approach to understanding the conformational propensities of isolated protein domains.

Project Description:

Objectives:

1) To prepare antibodies specific for particular proteins.

2) To use these antibodies to study conformational properties of the native protein as well as protein derivatives and fragments.

3) To use neurophysin-specific antibodies to isolate a proposed neurophysin biosynthetic precursor.

Methods Employed: Antisera are obtained by injection of appropriate antigen emulsions into rabbits, using the services of Meloy Laboratories for maintenance of rabbits, injection of antigen, and collection of sera. The presence of specific antibodies is tested by immunodiffusion, radioimmunoassay, and neurophysin-Sepharose affinity chromatography. The radioimmunoassays also are used to test, by competition analysis, the conformational nature of particular protein derivatives and fragments. The neurophysin-Sepharose approach is used for antibody isolation and purification. Covalent attachment of purified neurophysin antibodies in Sepharose is used for preparation of an immunoadsorbant, which is subsequently used in an affinity chromatographic routine to 'capture' cross-reacting material in various extracts, including those from bovine hypothalami. Adsorbed protein is examined by polyacrylamide gel electrophoresis, both conventional and in the presence of sodium dodecyl sulfate.

Major Findings: In the case of the neurophysins, specific antibodies can be prepared efficiently through the use of soluble poly Ala-poly Lys conjugates as antigens. These specific antibodies can be isolated and coupled to agarose matrices without loss of activity. The immunoadsorbants so prepared have been used to demonstrate the presence of neurophysin cross-reacting species of higher molecular weight in hypothalamic extracts. The conformational similarity of bovine neurophysins I and II has been verified by comparative affinity chromatographic properties of specific antibodies elicited against each.

Affinity chromatographic techniques also have been used successfully to isolate specific ribonuclease antibodies in pilot experiments.

Significance to Biomedical Research: The general use of poly Ala-poly Lys protein conjugates as antigens to enhance the production of antibodies for weakly immunogenic proteins is of general use in immunology. The preliminary success in obtaining and immobilizing neurophysin-specific antibodies will be of use in investigating the biosynthetic origin not only of neurophysin but of closely related peptide hormones oxytocin and vasopressin. Future studies with ribonuclease and thermolysin antibodies are aimed at the general goal of understanding the relationship between primary sequence information and the attainment of protein conformation.

Proposed Course of Action:

1) Systematic attempts to isolate pro-neurophysins using immobilized antibodies. Also, pursuit of conformational studies with neurophysin antibodies.

2) Purification of ribonuclease antibodies specific for the S-peptide region (residues 1-20) for use in characterizing semisynthetic sequence.

3) Pursuit of a collaborative effort with Dr. Angelo Fontana, University of Padova, to obtain and use thermolysin antibodies for characterizing large thermolysin fragments thought to contain isolated folding domains of the intact protein.

Publication:

Fischer, E.A., Curd, J.G., and Chaiken, I.M.: Preparation of Biologically Active Conjugates of Bovine Neurophysins and Other Polypeptides with Multi-(poly-D,L-alanyl)-poly-L-lysine and their Use to Elicit Antibodies. Immunochem., in press.

| SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space) | U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT | PROJECT NUMBER Z01 AM 25,008-14 LCB |
|---|---|---|

PERIOD COVERED

July 1, 1976 to September 30, 1977

TITLE OF PROJECT (80 characters or less)

Studies on the Dynamics of Protein Conformation

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

| | | | |
|---|---|---|---|
| PI: | Hiroshi Taniuchi | Chief, Section on Protein Conformation | LCB NIAMDD |
| | Diana S. Parker | Chemist | LCB NIAMDD |
| | Austine Davis | Chemist | LCB NIAMDD |

COOPERATING UNITS (if any)

LAB/BRANCH

Laboratory of Chemical Biology

SECTION

Protein Conformation Section

INSTITUTE AND LOCATION

NIAMDD:NIH; Bethesda, Md. 20014

| TOTAL MANYEARS: | PROFESSIONAL: | OTHER: |
|---|---|---|
| 2.9 | 0.9 | 2.0 |

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Precession photographs of crystalline Nuclease-T' are being taken at many levels of reciprocal lattice planes and the reflections thus recorded are being processed using a computer in order to obtain a difference electron density map between nuclease A and Nuclease-T.

The extra amino acid sequence contained in nuclease B, a possible precursor of nuclease A, has been established to be: Ser-Glu-Thr-Asp-Asx-Gly-Val-Asx-Arg-Ser-Gly-Ser-Glu-Asp-Pro-Thr-Val-Tyr-Ser where the two residues indicated by Asx are Asp in nuclease B1, and Asn in nuclease B3 and the first and the second Asx from the $NH_2$-terminus are Asp and Asn, respectively in nuclease B2. This extra sequence attached to the $NH_2$-terminus of nuclease A.

Saturation of Nuclease-(1-126) with ligands ($p^{dT}p$ and $Ca^{2+}$) decreases its intrinsic viscosity from 6.59 to 5.28 $cm^3/g$. The latter value for the intrinsic viscosity is equivalent to that of liganded nuclease B and clearly greater than that of liganded nuclease A. These results are compatible with the idea that liganded Nuclease-(1-126) may possess a conformation composed of ordered as well as flexible segments due to lack of cooperativity.

Project Description:

Objectives: To understand interatomic interactions in the three-dimensional structure of proteins in relation to the structures and functions of proteins.

Working Hypothesis: There would be a linkage network of interatomic interactions operative throughout the three-dimensional structure of native proteins, strengthening otherwise weak interatomic interactions (see the report of the previous year). This hypothetical linkage network of interatomic interactions in proteins would be responsible for the energy barrier of unfoulding and for cooperativity and would provide a way to transmit the effect of a change in interactions at one site of proteins to other sites.

Methods Employed and Major Findings: Our precession camera is now recording reflections of crystalline Nuclease-T' (see the reports of the previous years) up to 3 $A^{o}$ at-3 to $0^{o}$. The intensities of these photographically recorded reflections have been successfully measured with the aid of an automatic microdensitometer and the data thus obtained have been processed using a computor with the collaboration of Dr. Gerson Cohen, the Section on Molecular Structure, Laboratory of Molecular Biology, National Institute of Arthritis, Metabolism and Digestive Diseases. Thus, a difference electron density map between nuclease A and Nuclease-T' may be obtained in the near future.

Chemical synthesis of Nuclease-T-(6-48) has been resumed with the collaboration of Dr. Carlo DiBello, University of Padova. The synthesized fragment is being purified in our laboratory in the hope that semisynthetic Nuclease-T' (a complex of synthetic Nuclease-T-(6-48) and native Nuclease-T-(49-149)) may be crystallized for x-ray diffraction analysis.

The extra amino acid sequences of three nuclease B species have been established by A. Davis as summarized above. This extra sequence attached to the $NH_2$-terminus of nuclease A's flexible and does not interfere with the ordered structure or the function of the nuclease A portion. Thus, a possible precursor of nuclease, nuclease A, has now been found. Staphylococcal protease in the presence of ligands (deoxythymidine 3',5'-diphosphate and calcium ion), specifically cleaves the peptide bond linking the extra sequence to nuclease A.

A disordered fragment, Nuclease-(1-126) affords a valuable test object for our working hypothesis. Nuclease-(1-126) contains all the groups which interact with ligands in the three dimensional structure of nuclease A. Previously we observed that Nuclease-(1-126) preparations exhibit a low level of enzymic activity with heat denatured DNA as a substrate. Recently, Sachs et. al. (Sachs, D.H., Schechter, A.N., Eastlake, A., and Anfinsen, C.B. (1974) Nature 251, 242-244) demonstrated that this activity is intrinsic. Using a known synthetic substrate, deoxythymidine 3'-phosphate-$^{5}$ p-nitrophenyl phosphate, we have estimated that the dissociation constant of this substrate with Nuclease-(1-126) is approximately 300 times greater than with intact nuclease and that the maximum velocity of catalysis with the fragment is approximately 0.1% that with intact nuclease (see the report of the previous year).

We speculate that the conformation of nuclease-(1-126) is flexible since the hypothetical linkage network of interatomic interactions would not be operative in the native-like conformation of the fragment in contrast to intact nuclease A whose conformation is assumed to be stabilized by an extra stabiliz-

ing energy due to the hypothetical linkage network of interatomic interactions (see the working hypothesis). It would then follow that the conformational cooperativity would not be operative in the native-like conformation of the fragment.

If liganded Nuclease-(1-126) lacks conformational cooperativity, its conformation would be such that the groups interacting with the ligands would be arranged, in space, around the ligands and the rest of the polypeptide chain would be flexible. Thus, the conformation of liganded Nuclease-(1-126) would be composed of ordered and disordered portions. This deviation from a compact globular structure could be detectable by measurements of viscosity. On the other hand, if the free energy associated with the native-like conformation of the fragment is lower than that associated with any other conformation of the fragment and the ligands bind to the native-like conformation, then the liganded fragment would have a native-like conformation, a compact globular structure.

In order to show that a three dimensional structure composed of an ordered portion and a disordered portion exhibits a viscosity greater than that expected for a globular protein, we have used nuclease B, which contains a flexible extra sequence plus an ordered nuclease A portion (see above).

The results of viscosity measurements by D.S. Parker are as follows. As expected, nuclease B exhibits a significantly greater intrinsic viscosity (5.41 $cm^3/g$) than nuclease A (3.7 $cm^3/g$). The intrinsic viscosities of nuclease A and nuclease B do not change within experimental error upon addition of ligands. The intrinsic viscosity (6.59 $cm^3/g$) of nuclease-(1-126) in the absence of ligands is greater than that of nuclease B. In the presence of ligands at a concentration ($^{pdt}p$, 2 x $10^{-2}$ M; $Ca^{2+}$, $10^{-2}$ M) sufficient to essentially saturate Nuclease-(1-126), the intrinsic viscosity (5.28 $cm^3/g$) of the fragment decreases to the level of that of nuclease B. These results do not prove but are compatible with the idea that liganded Nuclease-(1-126) would have a conformation composed of ordered and flexible portions, a conformation different from the three dimensional structure of liganded nuclease A.

Significance to Biomedical Research and the Program of the Institute:
Interatomic interactions in proteins which are vital for maintaining the three-dimensional structures may be involved in the mechanism of enzymic activity and related to both protein-ligand and protein-protein (domain-domain, subunit-subunit) interactions and are not well understood. The studies of the complementing fragments of staphylococcal nuclease A have given insight into these interatomic interactions. Specifically, we have developed and are testing experimentally the hypothetical concept of the self-reinforcing three-dimensional linkage network of interatomic interactions which would provide the stabilizing energy for the three-dimensional structure and a way to transmit the effect of a change in interatomic interactions at one site in proteins to other sites. The mechanism of this hypothetical linkage network of interatomic interactions is unknown. An effort will be made to understand the physical reality of this linkage in future studies.

Proposed Course:
1) Determination of the atomic coordinates of Nuclease-T.
2) Crystallization and x-ray diffraction studies of semisynthetic Nuclease -T' and those having substituted residues.

3) Crystallization and x-ray diffraction studies of the inactive Nuclease -T analogue containing Nuclease-(6-43) and Nuclease-(49-149) and bound with substrates.

4) Crystallization and x-ray diffraction studies of type II complex (a second ordered complex of two nuclease fragments).

5) Themodynamic studies of the interaction of Nuclease-(1-126) with ligands.

6) NMR studies of interactions between two complemting fragments and those between fragments or ordered complexes and ligands.

7) Measurements of the energy barrier of unfolding of semisynthetic Nuclease-T containing substituted residues.

8) Studies of equilibration between type I and type II complexes containing substituted residues.

Publication:

Taniuchi, H., Acharya, A.S., Andria, G. and Parker, D.S. On the Mechanism of Renaturation of Proteins Containg Disulfide Bonds.
Friedman, M. (Ed.):
Advan. in Experimental Med. and Biology, Vol. 86A, Protein Crosslinking-Biochemical and Molecular Aspects
New York, Plenum, 1977, pp. 51-66.
Davis, A., Moore, I.B., Parker, D.S. and Taniuchi, H. Nuclease B: A Possible Precursor of Nuclease A, An Extracellular Nuclease of Staphylococcus Aureus. J. Biol. Chem. in press.

The publication which was reported as in press in the previous year:
Taniuchi, H., Parker, D.S. and Bohnert, J.L. Study of Equilibration of the System Involving Two Alternative, Enzymically Active Complementing Structures Simultaneously Formed From Two Overlapping Fragments of Staphylococcal Nuclease. J. Biol. Chem. 252: 125-140, 1977

| SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space) | U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT | PROJECT NUMBER ZO1 AM 25,009-03 LCB |
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PERIOD COVERED

July 1, 1976 to September 30, 1977

TITLE OF PROJECT (80 characters or less)

Mechanism of Renaturation of Reduced Lysozyme

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT


| | | | |
|---|---|---|---|
| PI: | A. Seetharama Acharya | Visiting Associate | LCB NIAMDD |
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COOPERATING UNITS (if any)

LAB/BRANCH

Laboratory of Chemical Biology

SECTION

Protein Conformation Section

INSTITUTE AND LOCATION

NIAMDD:NIH; Bethesda, Md. 20014

| TOTAL MANYEARS: | PROFESSIONAL: | OTHER: |
|---|---|---|
| 1.1 | 1.1 | 0 |

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)


Reduced partially carboxymethylated hen egg lysozyme (approximately 0.8 mol of [1-$^{14}$C] carboxymethyl groups) was air oxidised. The lower (native) and higher hydrodynamic volume forms fractions isolated contained radioactivity, approximately 35 and 65%, respectively. Ion exchange chromatography of the lower hydrodynamic volume forms yielded renatured lysozyme, two major radioacitve samples ($LH_C$ and $LH_D$) eluting at the positions of monocarboxymethylated lysozyme and two minor radioactive samples eluting at the positions of dicarboxymethylated lysozyme. Samples $LH_C$ and $LH_D$ contained 0.95 mol and approximately 0.5 mol of $^{14}$C carboxymethyl groups respectivity. The radioactive tryptic peptides of samples $LH_C$ and $LH_D$ indicated that all the eight isomers containing three, presumably, native disulfide bonds and one free and one carboxymethylated sulfhydryl group are formed during air oxidation of the reduced partially carboxymethylated lysozyme. Since in each one of these isomers the formation of one of the four native disulfide bonds is permanently blocked, it would follow that no one of the four disulfide bonds of native lysozyme is obligatory in the formation of other three native disulfide bonds.


PHS-6040

Project Description:

Methods Employed and Major Findings:

1) Monomeric nature of the higher hydrodynamic volume forms of lysozyme. Previous studies have shown that species of lysozyme containing non native disulfide bonds eluted from the columns of Bio gel P-30, at a position earlier than that of native lysozyme, but very much after the position expected for a dimeric species of lysozyme. In order to ascertain the monomeric nature of these species of lysozyme containing the non-native disulfide bonds, a detailed ultracentrifugal analysis of this material was undertaken. Determination of the molecular weight of this species by the sedimentation equilibrium method, clearly demonstrated that these are indeed monomeric, with an apparent molecular weight of 13,000. This establishes that a faster elution of these species on the Biogel columns in because of their enlarged molecular domain.

2) Reoxidation of reduced partially [1-$^{14}$C] carboxymethylated lysozyme: We have previously shown that during renaturation of reduced hen egg white lysozyme by air oxidation, three enzymically active isomers containing three, presumably, native disulfide bonds and one open disulfide bond between Cys 6 and Cys 127, or between Cys 64 and Cys 80 or between Cys 76 and 94 are formed. However the fourth isomer having the open disulfide bond between Cys 30 and Cys 115 was not found. In an attempt to study whether a three native disulfide bonded species of lysozyme containing open disulfide bond between the Cys 30 and Cys 115 also posses a lower hydrodynamic volume native-like structure, renaturation of reduced lysozyme having one of the eight Cysteinyl residues, presumably, randomly [1-$^{14}$C] carboxymethylated has been undertaken.

Reduced lysozyme was reacted with approximately 0.8 equivalents of [1-$^{14}$C] iodoacetic acid in 0.1M Tris/Acetate, pH 8.0 containing 6 M guanidine hydrochloride at 23$^{o}$C for 90 minutes. The reaction mixture was disalted and the desalted mixture containing reduced partially carboxymethylated lysozyme was incubated at 37$^{o}$ in 0.1M Tris/Acetate pH 8.0 containing 1.5mM β-mercaptoethanol at a protein concentration of 0.025 to 0.04 mg/ml for 36 hrs. for air oxidation. The air oxidised material was isolated by dialysis and lyophilization, and finally desalted by gel filtration.

On gel filtration of the reduced-partially-carboxymethylated oxidised lysozyme on Biogel P-30 columns, approximately 65 and 35% of the radioactivity eluted as the higher and lower hydrodynamic volume forms (LH forms). Ion exchange chromatography of the LH forms on Biorex-70 at pH 7.0 yielded four radioactive species, designated in the order of their elution from the column as $LH_A$, $LH_B$, $LH_C$ and $LH_D$ (containing 5,8, 65 and 23%, respectively, of the radioactivity of the LH forms ) and a non radioactive species, presumably the native lysozyme.

Samples $LH_A$ and $LH_B$ eluted at the positions of dicarboxymethylated lysozyme and have not been characterized further. $LH_C$ and $LH_D$ eluted at the positions of monocarboxymethylated lysozyme, and were enzymatically active (39 and 45% respectively). The sample $LH_C$ contained 0.95 moles of carboxymethyl groups and was essentially homogeneous in urea-acrylamide gel electrophoresis. Sample $LH_D$ contained 0.5 mol of [1-$^{14}$C] carboxymethyl group and gave two bands on urea-gel electrophoresis; the major band corresponded to the position of $LH_C$ and other to lysozyme. Thus it appears that $LH_D$ contained both mono-and non-carboxymethylated species.

To show that monocarboxymethylated species of sampel $LH_C$ and $LH_D$ contain three disulfide bonds and one for sulfhydryl groups and one $^{14}C$-carboxymethylated cysteine, the LH forms of reduced partially carboxymethylated-oxidised lysozyme were reacted with 10 fold molar excess of [1-$^{3}H$] iodoacetic acid in presence of 6M guanidine hydrochloride. Nearly one equivlaent of $^{3}H$-label was incorporated into the LH forms, clearly pointing out that these are three-disulfide bonded species.

Ion exchange chromatography of tryptic peptides of $LH_C$ indicated that all the tryptic peptides expected to contain carboxymethyl cysteines were radioactive. Interesting is the presence of radioactivity in the peptides T6, $T_9$ and $T_{15}$ containing carboxymethyl cysteine 30, 64 and 115 respectively. In the case of $LH_D$ almost all the radioactivities were present in peptide $T_{11}$, presumably the carboxymethylated cysteine is at position 80. On the basis of these results it is reasonable to assume that sample $LH_C$ is a mixture of seven of the eight isomers containing three, presumably, native disulfide bonds and one carboxymethyl cysteine and one free sulfhydryl group and that sample $LH_D$ contains one remaining isomer. When on the basis of the sets of three native disulfide bonds these eight isomers are grouped into the four structures, the calculated yields of the four structures are comparable to each other. These four structures must be formed from the respective species of the eight isomers of monocarboxymethylated reduced protein in which the formation of the respective native disulfide bond is permanently blocked. Thus none of the four native disulfide bonds appear to be obligatory in the formation of the three other native disulfide bonds.

3) Intermediates in the Renaturation of the Higher hydrodynamic volume forms of lysozyme: Previously we have shown that when the higher hydrodynamic volume form of lysozyme undergoing renaturation in the presence of β-mercaptoethanol is trapped with [1-$^{14}C$] iodoacetrate, lower hydrodynamic volume forms containing approximately 1 mole equivalent of carboxymethylated cysteine could be isolated. These lower hydrodynamic volume forms were chromatographed on Biorex-columns at pH 7.15 $\pm$ 0.05. Nearly 60% of the presumably three-disulfide bonded derivatives, chromatographed at a position corresponding to that of monocarboxymethylated lysozyme. Analysis of the tryptic peptides of this monocarboxymethylated lysozyme species indicated that it is a mixture of all the eight monocarboxymethylated, three disulfide bonded species of lysozyme. Thus it is clear that all the four isomeric forms of lysozyme with three native disulfide bonds and two free sulfhydryl groups are formed during renaturation of lysozyme and the pre-blocking the formation of the native disulfide bonds by alkylation is not necessary to generate these structures. Since these four isomers containing three native disulfide bonds, presumably, posses a native like spatial arrangement, the remaining two sulfhydryl groups would be proximal to each other, and hence the formation of the fourth disulfide bonds in each of the four structures would not be sterically hindered.

It has been shown earlier that the renaturation of randomly paired disulfide bonded, higher hydrodynamic volume form of lysozyme could also be catalyzed by thiol groups of the reduced lysozyme. Attempts have also been made to trap the intermediates during this renaturation process. The higher hydrodynamic volume forms were rearranged with equimolar amounts of reduced lysozyme at pH 8.0, 37°C for 10 mins. and the reaction was arrested by carboxymethylating the reaction mixture with [1-$^{14}C$] iodoacetate. The rearranged material

was fractionated by chromatography on Biogel P-30. The lower hydrodynamic volume forms thus isolated contained radioactive species indicating the presence of native like, incompletely oxidised species of lysozyme. Biorex chromatography gave three radioactive species and the native lysozyme. These three radioactive species were found to be the same as those obtained when the reoxidation misture of reduced lysozyme alone was trapped.

Only,three of the four isomeric structures of lysozyme containing three native disulfide bonds appear to be formed during the renaturation of higher hydrodynamic volume forms catalyzed by the thiol groups of reduced lysozyme. Since all the four isomeric structures are trapped when rearranged using β-mercaptoethanol, it appears that though there is a high flexibility in the order of disulfide bond formation during renaturation, this may be perturbed depending on the conditions under which the renaturation is carried.

In the conversion of the higher hydrodynamic volume form in the presence of an equimolar amount of reduced lysozyme, the amount of SH groups available to reduce the higher hydrodynamic volume forms is sufficient only to carry it to a stage of two disulfide bonded forms. It is possible, however that only one of four disulfide bonds in the non-native molecule is opened by intramolecular sulfhydryl-disulfide interchange with the thiols of other molecules and this molecule containing one open disulfide bond is then converted to the native form by sulfhydryl-disulfide interchange. This hypothesis assumes that any one of the four possible sets of lysozyme molecules containing three native disulfide bonds and one open disulfide are stable against sulfhydryl-disulfide interchange.

4) Reduction and Reoxidation of Hen egg white lysozyme containing carboxymethyl cysteines 6 and 127: - One of the native-like dicarboxymethylated derivative lysozyme isolated by trapping the reoxidation mixture of reduced lysozyme, is the one containing the carboxymethylated cysteine residues at positions 6 and 127 ($LH_1$). The ultraviolet absorption spectrum, tryptrophyl fluorescence emission spectrum, and the lower hydrodynamic volume nature of this derivative, as well as the presence of significant amount of lytic activity in this derivative suggests that a fold of the polypeptide chain close to that of the native lysozyme is maintained in this derivative even in the absence of the fourth disulfide bond. However the absence of the fourth disulfide bond in this derivatives makes the folding of the polypeptide chain less compact; i.e., more flexible, as seen with its elution behaviour on Biogel P-30 columns and its susceptibility to proteolysis with trypsin.

Fully reduced $LH_1$ could be renatured to $LH_1$ by air oxidation in the presence of 1.5 mM β-mercaptoethanol, clearly demonstrating that the reduced lysozyme, even after carboxymethylation at the two half cysteine residues at positions 6 and 127 contains all the information necessary to allow it to refold to the lower hydrodynamic volume lower conformational energy structure. However the efficiency of this renaturation appears to be only about 60%, whereas with the unmodified reduced lysozyme this is nearly 100%. This lower efficiency with the reduced $LH_1$ appears to be due to the decrease in the stability of the native format of disulfide bonds of $LH_1$ as compared with that of disulfide bonds of native lysozyme.

Significance to Biomedical Research: The results of the present study points out the high flexibility feature of the refolding process. It also

shows that even in the absence of any one of the four native disulfide bonds of lysozyme, the interatomic interactions of the molecules an sufficiently strong to contrain the lower hydrodynamic volume structure, characteristic of the native protein. The information of this kind will contributes to a more precise understanding of the detailed mechanism by which the tertiary structure of a protein is formed as programmed by its primary structure.

Proposed Course of Research:

1) Crystallization and x-ray diffraction studies of the three-disulfide bonded derivatives of lysozyme.

2) Look for possible derivatives of Lysozyme with lower hydrodynamic volume structures containing only two of the four native disulfide bonds and two open disulfide bonds.

3) Carboxypeptidase and amino peptidase digestion of the derivative carboxymethylated at half cysteine residues 6 and 127 in an attempt to get a complementing system to explore further the folding process of the reduced lysozyme.

Publication:

Acharya, A.S. and Taniuchi, H.: Formation of the Four Isomers of Hen Egg White Lysozyme containing three Native Disulfide Bonds and One Open Disulfide Bond. Proc. Nat. Acad. Sci. U.S.A. 74: 2362-2366, 1977.

Publication reported as in press in the previous report.

Acharya, A.S. and Taniuchi, H: A Study of Renaturation of Reduced Hen Egg White Lysozyme. J. Biol. Chem. 251: 6934-6946, 1976.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE
PROJECT NUMBER (Do NOT use this space)

U.S. DEPARTMENT OF
HEALTH, EDUCATION, AND WELFARE
PUBLIC HEALTH SERVICE
NOTICE OF
INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 AM 25,011-03 LCB

PERIOD COVERED

July 1, 1976 to September 30, 1977

TITLE OF PROJECT (80 characters or less)

Protein Unfolding by Fragment Exchange with a System Derived from Cytochrome c

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

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COOPERATING UNITS (if any)

LAB/BRANCH

Laboratory of Chemical Biology

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NIAMDD:NIH; Bethesda, Md. 20014

| TOTAL MANYEARS: | PROFESSIONAL: | OTHER: |
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| 1.1 | 1.1 | 0 |

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The (1-53)H:(54-104) complex an ordered complex formed by non-covalent interactions of the two fragments of cytochrome c formed by a limited trypsin digestion of the parent complex (1-65)H:(1-104) has been used in fragment exchange experiments to determine the activation free energy for unfolding of both oxidized and reduced forms of the complex. The slower rate of exchange of the ferrous form is consistent with its lack of susceptibility to trypsin digestion. The data support the concept of a dynamic equilibrium of folding and unfolding of cytochrome c under physiological conditions.



-0040
(Rev. 10-76)

Project Description:

Objectives: Estimation of the stabilization energy maintaining the structure of cytochrome c; finding a relationship between the stabilization energy and the atomic co-ordinates of the protein.

Methods Employed and Major Findings: The preparation of a biologically active, ordered complex from two overlapping fragments of cytochrome c has been described in previous annual reports (NIAMDD-LCB-12; 1974-5, 1975-6), in a "Report of Scientific Accomplishments", R. Hantgan and H. Taniuchi, April 28, 1976 and in J. Biol. Chem., 252, 1367-1374 (1977), R. Hantgan and H. Taniuchi. In summary, equimolar quantities of a cyanogen bromide heme fragment (1-65)H and apo protein (1-104) are mixed in solution at pH 7.8, the complex reduced to the ferrous state under anaerobric conditions, and the redundant portions of the complex removed by a limited trypsin digestion. The intact complex is separated from the trypsin cleaved peptides by gel filtration; then the individual apo and heme fragments separated by ion exchange chromatography in 8 M urea. Fluroescence quenching and lactate dehydrogenase assays of the recombined fragments indicated that the combinations (1-53)H: (56-104), (1-53)H: (54-104), (1-53)H:(40-104) and (1-53)H: (39-104) will regenerate many of the physical and biological properties of the intact protein, (1-104)H cytochrome c.

Following the time course of this digestion at 30, 60, and 90 minutes of trypsin cleavage indicated the final products to be (1-53)H: (54-104) and (1-53)H:(56-104).

Thus the system can be described by one type of complex with various lengths of redundant residues on the newly formed apo fragments, and that longer times of digestion can remove all redundant residues without perturbing the cytochrome c - like three dimensional structure of the resulting complex.

Therefore a large scale (600 mg of parent complex) preparation was carried out to obtain $\sim$ 33 mg (1-53)H: (54-104) and $\sim$ 10 mg (54-104) for subsequent fragment exchange experiments. The complex (1-53)H: (54-104) was incubated with a 50 fold excess of potassium $^{14}C$ cyanate at pH 6-7 for 1 h. After desalting, the mono-labelled derivative was separated by ion exchange chromatography. This complex, with a specific activity of $\sim 1.2 \times 10^{13}$ cpm/mole, exhibited spectral and biological properties which were indistinguishable from the non-radioactive complex.

Thus in principle, the exchange reaction (1-53)H: (54-104)-$^{14}C$ + (54-104) $\rightleftarrows$ (1-53)H: (54-104) + (54-104) -$^{14}C$ could be studied with this system. In practice a solution of the radioactive complex is mixed with an equimolar quantity of unlabelled apo fragment, incubated at 25.0°, quenched on ice, and separated into an apo fraction and a complex fraction by ion exchange chromatography at 6°. The cpm of the apo fraction and the cpm of complex/moles complex as determined spectrally, is used to measure the extent of the exchange reaction in a manner analogous to that used in the studies reported in the previous years with the Nase-T system. The rate of exchange was the same at a two fold excess of apo fragment, indicating that as in the Nase-T experiments, the process is first order and is, therefore, a measure of the unfolding rate of the (1-53)H: (54-104) complex. Using transition state theory, the free energy of activation is estimated as 24 kcal/mole for the unfolding re-

action at 25°, pH 7.

Similar measurements were carried out with the ferrous complex, but two steps are required to describe the exchange. From 0-240', exchange takes place 3-4 times slower in the reduced form whereas beyond 4h, the rate is at least 100 times slower than in the ferric form. (The first change may be an artifact of ascorbate reduction). In any case, exchange is overall, much slower in the reduced complex. Thus $\Delta G^{\ddagger}$ (reduced) $\geq$ 25.6 Kcal/mole. This observation is especially interesting as the x-ray crystallographic results of Dickerson and his colleagues (J.B.C., 252, 759-775, 1977) indicate no difference in conformation between ferric and ferrous tuna cytochrome c. This suggests that the reduced rate of unfolding of the ferrous (1-53)H:(54-104) complex accounts for its resistance to trypsin digestion, whereas the ferric form is readily cleaved. It also implies that the native cytochrome c structure exists in dynamic equilibrium with a small portion of unfolded protein, under physiological conditions.

Significance to Biomedical Research: Measurements to date have demonstrated that the energy barrier preventing the native structure of cytochrome c from unfolding can be measured by the fragment exchange technique. By chemical synthesis or hybrid complexes, it should be possible to determine the contribution that individual residues make to this stability, and to relate the primary sequence of a protein to its tertiary structure.

The concept of conformational motility is also emphasized in these experiments. i.e. if one observes the structure of cytochrome c by x-ray crystallographic methods or static spectral measurements, one sees a fixed structure. In contrast, fragment exchange allows the experimenter to measure the sum of the infrequent but large scale deformations in this structure which are missed in other techniques (except deuterium exchange). Thus the problems of dynamic structure and the energy barrier preventing these fluctuations can be studied by this technique in order to give a more complete picture of the structure of a protein in solution.

Proposed Course of Research:

1) Carry out fragment exchange at 30°, 35° to separate $\Delta G^{\ddagger}$ unfolding into its entropic and enthalpic contributions.

2) Crystallization and x-ray studies of the complex.

3) Prepare new complexes with desired sequence changes; measure activation parameters by exchange techniques.

Publication:

Hantgan, R.R. and Taniuchi, H.: Formation of a Biologically Active, Ordered Complex from Two Overlapping Fragments of Cytochrom C. J. Biol. Chem. 252, 1367-1374, 1977.

| SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space) | U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT** | PROJECT NUMBER Z01 AM 25,013-06 LCB |
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PERIOD COVERED
July 1, 1976 to September 30, 1977

TITLE OF PROJECT (80 characters or less)

Monospecific Antibodies as Probes of Hemoglobin Structure and Function and the Binding of Hemoglobin to Red Cell Membranes

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

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| | A.N. Schechter | Chief, Section on Macromolecular Biology | LCB NIAMDD |

COOPERATING UNITS (if any)

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| TOTAL MANYEARS: | PROFESSIONAL: | OTHER: |
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CHECK APPROPRIATE BOX(ES)

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Using non-precipitating monospecific antibodies as probes of conformation, we are studying structural differences of hemoglobins in solution. Hemoglobin $A_o$ and $A_2$ have been isolated, purified and carbamoylated with $K^{14}CNO$. Two specific subpopulations of anti-hemoglobin antibodies have been isolated by affinity chromatography using synthetic peptides corresponding to α(129-141) and δ(5-13). The first subpopulation will be used to study conformational changes at the carboxyl terminus of the α chain during oxygenation based on the difference in the binding affinity between $^{14}$[C]-oxy and $^{14}$[C]-deoxy hemoglobin. Differences will be used to examine mutant and truncated hemoglobins so as to better understand the structural changes occurring during the dynamic transition from the T (deoxy) state to the R (oxy) state. The second subpopulation will be used to measure hemoglobin $A_2$ levels in serum and in individual cells and to differentiate $A_2$ from other normal and mutant hemoglobins. In addition we are using the labeled normal hemoglobins to quantitate the binding of hemoglobin to red cell membranes and to determine the site of this binding.



PHS-6040

Project Description:

Objectives: For the last 5-6 years, this laboratory has been interested in the use of monospecific antibodies as probes to study conformational changes of proteins in solution. Initially work was begun with antibodies specific for staphylococcal nuclease fragments and later with antibodies directed against the amino terminal end of the β chain of sickle hemoglobin. At present we are directing our attention toward two other projects using antibody subpopulations as probes: 1) The detection of conformational differences in normal hemoglobin $A_o$ as it changes from the T state (deoxy) to the R state (oxy) upon oxygenation. 2) The quantitative measurement of hemoglobin $A_2$ ($\alpha_2\delta_2$), a normally occurring hemoglobin, by means of a radioimmunoassay using antibodies specific for a 9 residue peptide contained in the δ chain.

In addition, we have more recently begun both kinetic and equilibrium studies to determine the extent and specificity of the binding of labelled hemoglobin to red cell membranes.

Methods and Major Results:

Hemoglobin $A_o$

Hemoglobin $A_o$ has been chromatographically purified on DEAE Sephadex and isotopically carbamoylated with $K^{14}CNO$. The extent of modification has been determined independently by hydantoin analysis and the quantitation of homo citrulline as well as by specific activity. Although structurally similar to unmodified hemoglobin, the carbamoylated protein does have a slightly higher oxygen affinity. Thus, studies are now underway to determine the best conditions to alkylate hemoglobin using formaldehyde to form a Schiffs base which in turn is reduced with tritiated borohydride. In these studies we hope to obtain a specific activity approaching that of $I^{125}$ iodinated hemoglobin and to minimize functional changes.

Sheep immunized with $A_o$ hemoglobin have produced whole antisera which descriminates poorly between R (oxy) and T (deoxy) forms of hemoglobin as determined by quantitative precipitin curves. To improve this ability to differentiate between the two states we have used affinity chromatography to isolate a subpopulation of antibodies that are specific for the carboxyl terminus of the α chain of hemoglobin. It is in this region of the chain that the penultimate tyrosine and carboxyl-terminal arginine residues become exposed to the solvent in the oxy (R) state (having been protected in the deoxy (T) conformation).

To isolate such a monospecific subpopulation of antibodies we have synthesized a tridecapaptide homologous to the carboxyl terminus of the α chain α(129-141) by the Corley modification of Merrifield solid phase synthesis. This peptide was used to prepare a Sepharose-peptide affinity column that bound a monospecific set of antibodies.

These monospecific antibodies were then used in a direct binding assay with the various liganded states of isotopically labeled hemoglobin. The bound hemoglobin was separated from the free hemoglobin by a precipitating second antibody (burro-anti-sheep IgG) and the liganded state under assay conditions

was monitored spectrophotometrically by examining the hemoglobin in the supernatant of duplicate tubes. Particular care was needed in deoxygenating the dilute hemoglobin solution in order to prevent the formation of methemoglobin.

The antibodies bind with a high affinity ($10^7$ $M^{-1}$) to all of the liganded states of hemoglobin (oxy, carbonmonoxy, met) and essentially not at all with the unliganded or deoxy form. Further studies are underway to determine whether the monospecific antibodies can differentiate among the liganded states.

Hemoglobin $A_2$

An analogous strategy was used to prepare a specific assay for hemoglobin $A_2$. Whole antisera was obtained from sheep immunized with hemoglobin and this sera, as determined by quantitative precipitin curves, could differentiate well among $A_2$, $A_o$ and F hemoglobins. Monospecific antibodies are being isolated by affinity chromatography using a nonapeptide with the same sequence as $\delta(5\text{-}13)$. Both the ammonium sulfate precipitated fraction of the whole antisera and the monospecific antibodies obtained from the affinity chromatography will be used in a radioimmunoassay to specifically and sensitively quantitate hemoglobin $A_2$.

Hemoglobin Binding to RBC Membranes

Recently we have undertaken to study the binding of normal hemoglobin to red cell membranes. Red cells are washed with .15 M NaCl solution, hypotonically lysed, separated from leukocyte membranes containing proteases and resuspended in physiological buffers. Aliquots of the membranes are then incubated with $^{14}$[C] labeled hemoglobin and the bound hemoglobin is separated from the free hemoglobin by centrifugation at 27,000 x g. This separation is aided by addition of small amounts of ConA-Sepharose which agglutinates the membranes and improves the partitioning. We have found binding of hemoglobin to the membranes and binding can be inhibited by preincubation with unlabeled hemoglobin. We are now in the process of quantitating the kinetic and equilibrium parameters and ascertaining the biological specificity of the binding.

Significance: Elegant studies by Perutz and his colleagues in the 1960's have shown significant tertiary and quarternary changes in the conformation of hemoglobin upon oxygenation. We hope that an assay using monospecific antibodies to oxyhemoglobin in solution will complement these crystallographic studies and give further information about the dynamics of this conformational shift. We also plan to use the Oxy-Deoxy assay to look at mutant hemoglobins with abnormal oxygen affinity in order to better define structure-function relationships.

Antibodies specific to hemoglobin $A_2$ will have direct clinical application in measuring levels of $A_2$ in the fetus and in the adult, as well as distinguishing hemoglobin $A_2$ from other hemoglobins (such as hemoglobin C) which have a similar electrophoretic mobility. We also will use this assay to quantitate hemoglobin in individual cells in order to examine at the molecular level the synthesis of hemoglobin $A_2$.

The phenomena of hemoglobin binding to red cell membranes has not been studied quantitatively. As yet there is no data on the equilibrium or kinetics of this binding process. The site of binding is as yet undefined, as is the

effect of this binding on red cell membrane permeability and deformability. The role that this binding may have on the normal aging process or on the hemolysis seen with some mutant hemoglobins is also an open question.

Proposed Course:

Hemoglobin $A_o$: To further validate the assay we would like to show that it has functional specificity (by the effect of the antibodies on the oxygen affinity of hemoglobin as well as structural specificity (by its decreased ability to bind des-Arg-Tyr) hemoglobins). We will then use the assay to examine mutant hemoglobin with abnormal oxygen affinities.

Hemoglobin $A_2$: We plan to continue the development of the assay and use it to examine the molecular control of the biosynthesis of this hemoglobin, as well as in its clinical detection.

Hemoglobin-RBC Membrane Binding: We plan to use the assay to study the specificity and significance of the interaction of hemoglobin with red cell membranes.

| SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space) | U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT | PROJECT NUMBER Z01 AM 25,014-03 LCB |
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PERIOD COVERED

July 1, 1976 to September 30, 1977

TITLE OF PROJECT (80 characters or less)

Functional Properties of Immune Response Gene Regulation of Antibody Response to Nuclease

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

Principal Investigators:


Alan N. Schechter — Chief, Sec. on Macromolecular Biology — LCB NIAMDD


COOPERATING UNITS (if any)

(Immunology Branch, NCI (D.H. Sachs and G.M. Shearer) Metabolism Branch, NCI (Berzofsky) Laboratory of Immunology, NIAID (R.H. Schwartz)

LAB/BRANCH

Laboratory of Chemical Biology

SECTION

Section on Macromolecular Biology

INSTITUTE AND LOCATION

NIAMDD:NIH; Bethesda, Md. 20014

| TOTAL MANYEARS: | PROFESSIONAL: | OTHER: |
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CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)


The ability to raise an immune response to specific antigens has recently been shown to be under genetic control, linked to the major histocompatibility complex (MHC) in several species studied. In the mouse, a number of synthetic antigens have been found subject to this H-2 linked control, but only a few natural globular proteins have been studied as antigens, among these, staphylococcal nuclease. We have now tried to delineate the individual antigenic determinants on this protein which are responsible for this genetic control of the immune response in inbred strains of mice, in order to better understand the mechanisms involved. We have found that a single peptide fragment of the protein appears to be under the same control as the whole molecule. Furthermore when antibodies made to the whole nuclease molecule are studied for their specificity, we have found that the H-2 linked immune response genes can control the response to individual determinants on the same molecule independently of one another, and therefore may be involved in the selection of specific B lymphocytes rather than only T cells.

Project Description:

Objectives: It has previously been shown that immune response genes (Ir genes) which map within the major histocompatibility couples (H-2) of mice determine the ability of various inbred strains of mice to response to diverse antigens with antibody production and/or cellular immunity. These are apparently a separate level of regulator genes, which have the ability to recognize and distinguish a broad range of immunogenic determinants. In synthetic polypeptide antigen studies these determinants have generally been identified with backbone or "carrier" determinants. However, natural protein antigens have not been widely studied, and it is not clear what part of the molecule might serve this "carrier" function, or how it might be recognized. One objective of this study is to understand these mechanisms. Previous investigations have shown that the antibody response to staphylococcal nuclease in mice is under H-2 linked Ir gene control. In order to understand the mechanisms of Ir gene control of the response to natural globular protein antigens, we have undertaken to delineate the response to nuclease by a study of the course of the response with time and by using peptide fragments representing subregions of the molecule in five inbred strains of mice.

Major Results and Methods Employed:

1. Using an assay for antibodies to whole nuclease by inhibition of enzymatic activity, we have found that the strains A/J and B10·A, which share the same high responder $H\text{-}2^a$ haplotype but differ at other genetic loci, have consistently different levels of anti-nuclease response even with multiple boosting. This result implies non-H-2-linked control of the magnitude of the response as well as the H-2 linked control.

2. The B10 strain ($H\text{-}2^b$), a low responder in the initial response, eventually produces antibody levels comparable to the high responder B10·A congenic strain after several boosts, when inhibition of whole nuclease is the assay.

3. Using newly developed radioimmunoassays for antibodies binding to $^{14}C$-labeled fragments of nuclease corresponding to residues 99-149 and 1-126 (prepared as described in the previous report), it was found that the concentration of antibodies made to whole nuclease which bound to the 1-126 fragment was approximately the same in the two congenic strains B10 and B10·A. In contrast, a markedly lower concentration of the antibodies bound to the 99-149 fragment in the B10 than in the B10·A strain. Therefore, since the strains differ only at H-2, an H-2 linked Ir gene can control the response to individual determinants on the same antigen molecule independently of one another, contrary to expectations.

This result also explains the delayed response of B10 mice compared to B10·A when binding to whole nuclease was measured, since the B10 mice never produced as much antibody to the 99-149 region, but presumably were making antibodies to weaker determinants in other regions of nuclease after multiple boosts.

4. The high responder A/J and SJL mice were also found to make a significant response to the 99-149 region of nuclease when immunized with the whole molecule.

5. When the individual fragments themselves were used as immunogens in five strains of mice (3 high and 2 low responders), and sera from individual mice tested individually using groups of 8-12 mice per strain per immunogen over 3 immunizations, it was found that response to the 99-149 fragment followed the same pattern as that to whole nuclease. However, the B10, which was a low responder to nuclease and showed increased response with boosting, was a nonresponder to the 99-149 fragment, even after 3 immunizations. In contrast, all of the strains (except the other low responder, DBA/1) responded to the 1-126 fragment. A third fragment, 6-48, used as a control for its similarity to 99-149 in size and charge, was a poor immunogen in all 5 strains studied. Thus, the response to one peptide fragment of nuclease appears to be under the same genetic control as that to whole nuclease, despite the largely random conformation of the peptide.

6. The T-lymphocyte proliferative response to nuclease was measured using the mouse peritoneal exudate, T-lymphocyte enriched cells (PETLES) assay. Genetic control by MHC-linked Ir genes could be discerned. Genetic mapping initially localized the gene to the I-B subregion, but some results suggest more complex control. Indeed, the recombinant data suggest control involving several Ir genes in the I, K, and D regions.

Significance to Biomedical Research: The mechanism of the genetic control of the immune response is important in understanding the steps leading from immunization with an antigen to the final production of antibodies. It is also important in understanding the diseases whose pathogenesis is partially immunological - such as the autoimmune diseases and immune deficiency states, including the specific immune deficiency seen in cancer. Our results have the following specific implications about this genetic control: 1) The additional non-H-2 linked control of the antibody response suggests other independent mechanisms of control which may be studied. 2) The finding of a fragment of nuclease which may be under the same control as the whole molecule opens up the possibility of further delimiting the regions which determine the immune response recognition by the use of other natural and synthetic peptides. 3) If the interpretation is correct that H-2 linked genetic control can be the same for a random conformation fragment as for native nuclease, then this suggests either a) that the receptors involved show less conformational specificity than the antibodies ultimately produced (which distinguish sharply between native and random conformations) or b) that the key determinant recognized is short or flexible enough to not differ significantly between the random and native structures. 4) The result that the response to two different determinants on the same molecule can be controlled independently by H-2 linked genes in strains that share the same immunoglobulin structured genes, suggests that H-2 linked Ir gene control is exerted at the level of selection of specific B cells, and raises serious doubt about the popular model which states that the presence of T cells which can recognize a single "carrier" determinant on the molecule is sufficient to allow triggering of B-cell response to any determinant on the same molecule. 5) The study of genetic control at the T cell level allows one to discern the role of specific genes on the regulation of the immune response. The careful study of nuclease and its fragments in this way should yield important information about these factors for globular proteins.

Proposed Course of Project: This project has been discontinued for the present.

Publications:

Berzofsky, J.A., Schechter, A.N., Shearer, G.M., and Sachs, D.H.: Genetic Control of the Immune Response to Staphylococcal Nuclease. III. Time Course and Correlation Between the Response to Native Nuclease and the Response to its Polypeptide Fragments. J. Exp. Med. 145, 111-122 (1977).

Berzofsky, J.A., Schechter, A.N., Shearer, G.M., and Sachs, D.H.: Genetic Control of the Immune Response to Staphylococcal Nuclease. IV. H-2-linked Control of the Relative Proportions of Antibodies Produced to Different Determinants of Native Nuclease. J. Exp. Med. 145: 123-135 (1977).

Berzofsky, J.A., Schwartz, R.H., Schechter, A.N., and Sachs, D.H.: H-2 Linked Control of the Antibody and Cellular Immune Response to Nuclease at the Level of Individual Regions of the Molecule. McDevitt, H.S., Paul, W.A., and Benacerraf, B. (Eds.) Proceedings of the Third Ir Gene Workshop, in press (1977).

| SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space) | U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT** | PROJECT NUMBER Z01 AM 25,016-04 LCB |
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PERIOD COVERED
July 1, 1976 to September 30, 1977

TITLE OF PROJECT (80 characters or less)

Immunological and Structural Studies of Human Fetal Hb

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

| | | | |
|---|---|---|---|
| PI: | A. Eastlake-Dean | Chemist | LCB NIAMDD |
| | A.N. Schechter | Chief, Section on Macromolecular Biology | LCB NIAMDD |

COOPERATING UNITS (if any)

Department of Biochemistry, Washington University, St. Louis, Mo.
(Dr. K. Thomas)

LAB/BRANCH
Laboratory of Chemical Biology

SECTION
Macromolecular Biology Section

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20014

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Human fetal hemoglobin (HbF) differs from HbA in both functional and structural aspects. These differences are evidenced by the weaker affinity of HbF for 2,3 diphosphoglyceric acid, and by the non-participation of HbF in gel formation with HbS. The molecular basis of these differences is not clear. There are 39 amino acid differences between the γ chains of HbF ($\alpha_2\gamma_2$) and the β chains of HbA ($\alpha_2\beta_2$), several of which are found in the 2,3 diphosphoglyceric acid bind[illegible] site. A number of other amino acid substitutions are clustered in one exposed helical region of the γ chain. We are using solid phase synthesis to reproduce this helical region. Immunoabsorption with this synthetic peptide of an antiserum to HbF is being used in an attempt to obtain non-precipitating antibodies specific for this region which do not crossreact with HbA or other hemoglobins. A radioimmunoassay using [$^{14}$C]-carbamoylated HbF will be used to assess the specificity of binding. We have also obtained crystals of met-HbF of suitable size for X-ray diffraction studies. The specific antibodies and X-ray studies will give infor[illegible]tion on the conformational and surface structural characteristics of HbF. In addition the antibodies may be useful in quantitating small amounts of HbF in standard laboratory procedures and in prenatal diagnosis of hemoglobinopathies

Project Description:

Objectives: Immunologic studies of staphylococcal nuclease and, subsequently, human hemoglobin in our laboratory have used several new approaches. First, synthetic peptides have been produced rapidly and in good yield by a solid phase method. Second, these peptides, representing a portion of the amino acid sequence of the protein, have been insolubilized by attachment to an inert support and used to select from an antiserum to the protein those antibodies which specifically bind in the region of the protein represented by the peptide sequence. Third, a radioimmunoassay has been used to demonstrate the specificity of the binding of such a restricted antibody population by competition between the peptide and the intact protein, and by demonstration that protein molecules with structural alterations (i.e. an amino acid substitution) in the region of antibody specificity do not bind the antibodies.

Human fetal hemoglobin has 39 amino acid differences from HbA some of which are clustered in limited areas of the surface of the molecule. Other substitutions are in the binding site for 2,3-diphosphoglyceric acid. Both the surface interactions and the 2,3-DPG binding affinity of HbF are different from those of HbA. The goal of this project is the isolation of antibodies specific for HbF and their use as probes of the conformation and surface structural features of this molecule. To this end a study of the crystal habit of HbF is also being undertaken.

Methods: The isolation of human HbF from umbilical cord blood samples and sickle cell anemia patients with high levels of HbF has been carried out by standard ion-exchange chromatographic procedures. Peptides have been prepared using a rapid modification of the Merrifield solid-phase method. Gel filtration has been used to purify the peptides. They have been analyzed for completeness of each coupling step by the 2,hydroxynaphthaldehyde method and for overall purity by fingerprinting and amino acid analysis.

Peptides have been coupled to Sepharose beads by CNBr activation. Antisera to HbF was raised in sheep. Fractionation of the antisera on peptide-Sepharose columns will be performed. HbF was labelled with $K^{14}CNO$ for use in a radioimmunoassay which will be developed to assess the specificity of binding of the fractionated antibodies.

Crystallization trials have been carried out using a vapor diffusion method and the conditions described by Perutz for the crystallization of human HbA. Crystallization of met-HbF has been attempted in order to produce a crystal of suitable durability for X-ray study.

Findings: Highly purified HbF has been isolated from cord blood samples. Peptides representing specific regions of the molecule have been synthesized. Crystals of met-HbF have been obtained at 4°, 2.5 M phosphate buffer and 4% HbF concentration. Typical crystal sizes are 1 x 0.6 x 0.6 mm.

Significance to Biomedical Research: The $\gamma$ chain of HbF may have arisen by gene duplication from the $\beta$ chain of HbA. No differences which might account for the selective advantage of HbF over HbA have been found. Studies of the conformation and surface structural characteristics of HbF may shed

light on these differences. This can be approached by using specific antibodies to HbF and through crystal structure studies. In addition, antibodies specific to HbF may be used to quantitate small amounts of HbF replacing methods now in use which are not highly quantitative. These antibodies may be useful in prenatal diagnosis of abnormalities of hemoglobin production and sickle cell disease, and in studies of the differentiation of erythroid cells.

Proposed Course: Peptides representing specific sequences exposed on the surface of HbF in regions where there are differences from HbA will be used as immunoabsorbants to isolate antibodies specific to HbF which do not cross-react with HbA. [$^{14}$C]-carbamoylated HbF will be used in a radioimmunoassay to determine the specificity of the antibodies. The assay will be used to quantitate HbF in mixtures of hemoglobins both in whole blood and in individual red cells. We will also use the assay in immunochemical studies of the nature of the HbF molecule as an antigen.

The collection and analysis of diffraction data on the crystals of HbF will continue. The space group will be determined on a precession camera. If these studies are successful we may pursue an X-ray analysis to high resolution.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE
PROJECT NUMBER (Do NOT use this space)

U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE
PUBLIC HEALTH SERVICE
NOTICE OF
INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 AM 25,017-05 LCB

PERIOD COVERED
July 1, 1976 to September 30, 1977

TITLE OF PROJECT (80 characters or less)

Immunochemical Studies of RNA

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

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|---|---|---|---|
| PI: | D. Eilat | Visiting Research Fellow | LCB NIAMDD |
| | A.N. Schechter | Chief, Section on Macromolecular Biology | LCB NIAMDD |

COOPERATING UNITS (if any)
Arthritis and Rheumatism Branch, NIAMDD (Dr. Alfred Steinberg)
Laboratory of Microbial Immunity, NIAID (Dr. Richard Asofsky)

LAB/BRANCH
Laboratory of Chemical Biology

SECTION
Macromolecular Biology Section

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20014

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

We have studied single stranded (ss) RNA binding immunoglobulins in the sera of patients with systemic lupus erythematosus as part of a project to obtain antibodies with conformational specificity for nucleic acids. The antibody population that bound polyribonucleotides and the population that bound polydeoxyribonucleotides appear to be distinct. The anti ss-RNA antibodies preferentially bound native ssRNA (as compared with synthetic polyribonucleotides) with an increasing affinity for the higher molecular weight RNA's. We have also developed an assay for detection of specific RNA antibodies produced in vitro by spleen cells from old female NZB/NZW F1 mice. This assay enables one to test the effect of different therapeutic agents at the immediate site of antibody synthesis.



PHS-6040

Project Description

Objectives: Antibodies to native nucleic acids are difficult to elicit in experimental animals, but can be found with great frequency in the sera of patients with systemic lupus erythematosus (SLE) and NZB/NZW F1 mice that develop an autoimmune disease resembling human SLE. The exact chemical specificities of these nucleic acid reactive antibodies and their relation to the autoimmune disease are not yet fully understood. We have previously studied the properties of tRNA antibodies in NZB/NZW mice sera, as part of a project to obtain antibodies with conformational specificity for nucleic acids. This work has now been extended to RNA reactive antibodies in sera of SLE patients and to the production of RNA autoantibodies by mouse spleen cells cultured in vitro.

Methods Employed: Radioactive $^{125}$I-RNA was prepared by iodination of MS2 phage RNA in the presence of thallic perchlorate. Radioactive $^{3}$H-tRNA was prepared by isolating tRNA from Qβ E. coli cells grown on $^{3}$H-uridine containing medium. The radioassay used for detection of antibodies utilized nitrocellulose filters for isolation of RNA·antibody complexes. Ribonuclease activity was removed from serum by charcoal treatment or by gel filtration. Mouse and human IgG immunoglobulins were isolated by ammonium sulfate precipitation, DEAE-cellulose chromatography and by gel filtration. Human sera were obtained from the tissue bank of the Arthritis and Rheumatism Branch of the NIAMDD. NZB and NZW mice were obtained from colonies maintained at the NIH. Active lymphocytes were obtained from spleens of 8 month old female NZB/NZW F1 mice or normal mice and cultured for short periods (24-48 hrs) in a $CO_2$-incubator. The culture supernatants were then collected and processed to obtain the active IgG antibodies.

Major Findings:

1) Antibodies that bound single stranded (ss) RNA occurred with high frequency in sera of patients with SLE but not in sera of patients with other connective tissue diseases that were tested. The antibody population that bound polyribonucleotides and the population that bound polydeoxyribonucleotides appeared to be distinct.

Competition experiments showed that the anti-ssRNA antibodies preferentially bound native ssRNAs (as compared with synthetic single and double stranded polyribonucleotides) with an increasing affinity for the higher molecular weight ssRNAs. No indication of conformational specificity of the antibodies was found. The anti-tRNA population was of restricted heterogeneity Sips index 0.83) and bound tRNA with an average Ko of $9 \times 10^{6}$ 1/mole at 4°. The anti-MS2 RNA population was much more heterogeneous (Sips index 0.67) and bound MS2 RNA with Ko of about $3 \times 10^{9}$ 1/mole at 4°.

2) An assay was developed, to detect minute amounts of specific autoantibodies, produced in vitro by spleen lymphocytes from old female NZB/NZW F1 mice. The assay utilizes highly radioactive $^{125}$I-RNA and IgG obtained from culture supernatants, and purified by DEAE cellulose chromatography. No antibodies with RNA specificity were produced by spleen cells from various normal mice (Balb/c, DBA/2) or by young female NZB/NZW F1 mice. Treatment with RNA and protein synthesis inhibitors showed that the specific RNA antibodies from

old NZB/NZW spleen cells are synthesized de novo during incubation in vitro and do not result from spillover of serum antibodies.

Significance to Biomedical Research: This project may lead to new information on the origin, development and genetics of certain autoimmune diseases, particularly systemic lupus erythematosus. It is generally accepted that only a small portion of the circulating nucleic acid-reactive antibodies in SLE patients are found as precipitates in the kidney glomeruli. It will be of great interest to explore the exact chemical specificities of the circulating and deposited antibodies. The new in vitro assay for production of specific autoantibodies in mice will enable one to test the effect of different therapeutic agents directly at the site of antibody synthesis.

Proposed Course of Project:

1) An attempt will be made to isolate SLE antibody populations specific for single nucleotides by affinity chromatography.

2) The relatively homogeneous NZB/NZW anti-tRNA antibodies, already purified to a substantial extent will be used to elicit anti-idiotype antibodies (in rabbits) that will be tested as blocking agents in the new in vitro assay.

Publication:

Eilat, D., Di Natale, P., Steinberg, A.D., and Schechter, A.N.: Properties of tRNA Specific Antibodies from NZB/NZW Mice. J. Immuno. 118, 1016-1024 (1977).

| SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space) | U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT** | PROJECT NUMBER Z01 AM 25,019-02 LCB |
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PERIOD COVERED
July 1, 1976 to September 30, 1977

TITLE OF PROJECT (80 characters or less)

Nucleic Acid Chromatography and Chemical Modification

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

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|---|---|---|---|
| PI: | K. Thomas | Guest Worker | LCB NIAMDD |
| | A.N. Schechter | Chief, Section on Macromolecular Biology | LCB NIAMDD |

COOPERATING UNITS (if any)

Montreal Clinical Research Institute (Dr. P. Schiller)

LAB/BRANCH
Laboratory of Chemical Biology

SECTION
Macromolecular Biology Section

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20014

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The ethidium cation of the salt ethidium bromide has been previously shown by others to be a sequence specific nucleic acid intercalating agent. We have demonstrated the interaction of gel bound ethidium ion into nucleic acid double helices by a variety of biophysical techniques including fluorescence. Preliminary experiments indicate that crude transfer ribonucleic acid can be fractionated to some degree on this column using elution with a linear sodium chloride gradient. It has also been found that $tRNA^{Phe}_{E.\ coli}$ can be selectively reacted at the X-base with N-hydroxysuccinimide esters of fluorescent compounds. This reaction has been studied as a source of information about the function of this group and as a spectroscopic probe of tRNA structure.

Project Description:

Objectives: Many planar polycyclic aromatic compounds, including those of the phenanthrilic class such as the ethidium cation, have been demonstrated to intercalate (i.e. insert) between adjacent base pairs in nucleic acid double helices. Recent solution results indicate that the ethidium cation can discriminate various intercalation sites that are formed by different combinations of base pairs in such helices. This sequence specific recognition of double helical polynucleotides is thus a potentially useful physical basis for the fractionation of these molecules. Therefore, we have begun an investigation of the potential use of the ethidium cation covalently linked to agarose to fractionate nucleic acids.

The specific modification of tRNA molecules affords a way to examine the functions of regions of the molecule as well as a source for spectroscopic probes of reactions which involve tRNA.

Methods Employed: Cyanogen bromide activated agarose was reacted with 3,3'-diaminodipropylamine followed by succinic anhydride to produce a "spacer arm" with a terminal carboxylic acid group. The ethidium cation was covalently linked to this carboxyl group by utilizing a water soluble carbodiimide mediated coupling. A column that was packed with this material has been used to bind a crude mixture of E. coli transfer ribonucleic acid (tRNA) species. The ethidium column was eluted with a linear sodium chloride gradient at room temperature. The fractions were assayed by a radioactive tRNA aminoacyl charging technique. The complex of tRNA with the ethidium gel was also measured by both direct spectrophotometric and fluorescence measurements of the gel. Equilibrium partition measurements were made on a tRNA solution in the presence of the ethidium gel.

$tRNA^{Phe}_{E.\ coli}$ was labeled with the N-hydroxysuccinimide esters of 1-dimethylaminonaphthalene-5-sulfonyl glycine and N-methylanthranilic acid through reaction with the amino acid moiety of its X-base, whereby yields of 66% and 24%, respectively, were obtained.

Major Findings: E. coli methionine and phenylalanine tRNAs are clearly separated from each other by sodium chloride linear gradient elution. The sodium chloride sensitivity of the ethidium-nucleic acid complexes in the early eluting methionine tRNA and the latter eluting phenylalanine tRNA fractions were compared in solution after removal of the elution salt by dialysis. The ethidium complex of the earlier eluting fraction was dissociated by a lower concentration of sodium chloride than the latter eluting fraction which is thus consistent with the behavior of these fractions on the column. The fractionation was demonstrated to be a result of the bound ethidium interacting with the nucleic acid since a control column, identical to the ethidium column except that the ethidium group at the end of the 'spacer arm' was replaced by an ethylamine group, did not retard the tRNAs at salt concentrations well below those necessary to elute the ethidium column. The basis for fractionation was shown to involve intercalation of the covalently bound phenanthriline ring into the nucleic acid double helical structure since the ethidium fluorescence was enhanced upon binding tRNA, a physical parameter previously determined to be indicative of the intercalation complex. Further-

more, fluorescence enhancement, absorbance maximum red shift, equilibrium partition binding and the dynamic flow binding of the tRNA to the gel paralleled each other in titration of complex formation with tRNA and subsequent complex dissociation by sodium chloride.

The purified dimethylaminonapthalene-sulfonate derivative could not be aminoacylated and was found to be a strong competitive inhibitor of phenylalanine-tRNA synthetase [$K_i$-8x10$^{-7}$ M]. The N-methylanthranilyl derivative could be charged to an extent of 5% as compared to native $tRNA^{Phe}$. The fluorescence emission spectra of the derivatives are indicative of a slightly hydrophobic environment for both fluorophores. The results suggest that the integrity of the polar amino acid group of the X-base is required for the maintenance of the biologically active conformation.

Significance to Biomedical Research: The principle of intercalation chromatography may be useful for the fractionation of nucleic acids. Furthermore, since many intercalating compounds, including salts of the ethidium cation, are used as drugs and are thought to mediate their effect through interaction with nucleic acids by intercalation the elucidation of the specificity of this interaction is necessary to the understanding of the detailed mechanism of action of these compounds. Also, since it has been proposed that surface aromatic amino acids on proteins that interact with nucleic acids may participate in intercalation complexes with the nucleic acid double helices, the investigation of the discriminatory potentials of the intercalation process could have relevance to the more general area of specific protein-nucleic acid recognition which is of crucial importance for normal cell function in the defense of cells against foreign genetic material.

This new, derivatized species of tRNA will be useful for studying the conformation in solution of this molecule, as well as its interactions with other molecules and subcellular organelles during protein biosynthesis.

Proposed Course of Project: This project has been discontinued for the present.

Publications:

Schiller, P.W. and Schechter, A.N.: Covalent Attachment of Fluorescent Probes to the X-base of E. coli Phenylalanine Transfer Ribonucleic Acid. Nucleic Acids Res., in press.

Thomas, K.A. and Schechter, A.N.: Evolutionary and Chemical Constraints on the Folding of Proteins. Goldberger, R.F. etal. (Eds.) Biological Regulation and Development, Plenum Press, in press.

| SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space) | U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT | PROJECT NUMBER Z01 AM 25,020-02 LCB |
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PERIOD COVERED
July 1, 1976 to September 30, 1977

TITLE OF PROJECT (80 characters or less)
Radioimmunoassay of Creatine Kinase: A Model for Radioimmunoassay of Human Serum Enzymes

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: Alan N. Schechter Chief, Section on Macromolecular Biology LCB NIAMDD

COOPERATING UNITS (if any)
Clinical Pathology Dept., Clinical Center (Dr. Andre C. van Steirteghem and Dr. Mark H. Zweig)

LAB/BRANCH
Laboratory of Chemical Biology

SECTION
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NIAMDD, NIH, Bethesda, Maryland 20014

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SUMMARY OF WORK (200 words or less - underline keywords)

Human serum enzyme assays for clinical diagnosis usually make use of measurements of catalytic activity. In many situations, this may not be ideal; e.g. isoenzymes reflecting different damaged tissues may be indistinguishable or inactive enzymes may be released. Radioimmunoassay offers a very sensitive and specific way in which to measure enzyme concentration and to distinguish isoenzymes. Creatine kinase was chosen as a model for this approach because of its clinical importance and its simple isoenzyme distribution. The enzymes from human skeletal muscle (MM) and brain (BB) have been purified, used as immunogens to raise antibodies, and labeled with $^{125}$I. Radioimmunoassays for each of these enzymes were developed and were found to correlate well with standard enzyme assays in clinical use. An attempt is now being made to develop a specific assay for the hybrid MB species.



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Project Description:

Objectives: There are theoretical reasons for believing that direct measurement of enzyme molecule concentrations in human serum may have significant advantages over measurements of enzyme activity for diagnostic studies. Enzyme assays are insensitive to fragments of molecules or other inactive species and do not distinguish among isoenzymes that may come from different tissues. Radioimmunoassay has the potential of great sensitivity and specificity, including, frequently, the ability to discriminate among isoenzymes. Further, the immunological characteristics of a protein may be detectable in incomplete or inactive molecules.

In order to begin a study of the clinical potential of radioimmunoassay of human serum enzymes, we have purified creatine kinase and developed a radioimmunoassay. This radioimmunoassay is now being applied in several clinical applications.

Methods Employed: Human brain and skeletal muscle were homogenized, extracted, and the creatine kinase isoenzymes were purified by ethanol and ammonium sulphate precipitation, anion exchange chromatography, and gel filtration. The purified muscle (MM) and brain (BB) enzymes were characterized by disc gel electrophoresis and amino acid analysis.

Sheep and rabbits were immunized by repeated injection of these purified enzymes in Freund's adjuvant. Antibody production was followed by Ouchterloy, quantitative precipitation and immunoinhibition assays.

The MM and BB enzymes were labeled with $^{125}I$ by the Bolton-Hunter acylation reagent and purified by gel filtration. A double antibody radioimmunoassay was developed for the primary antibodies with burro-anti-sheep and sheep-anti-rabbit gamma globulin.

Major Findings: The enzyme purification steps yielded about 10 mg of essentially pure muscle creatine kinase and about 40 mg of essentially pure brain creatine kinase. The enzymes behaved as single bands on disc gel electrophoresis and the amino acid composition was similar to that previously reported for these enzymes. The molecular mass of each was about 80,000 daltons as determined by SDS-poly-acrylamide electrophoresis. Antibodies to each were obtained in good titer. $^{125}I$ labeling of each enzyme was accomplished with the Bolton-Hunter reagent and a double-antibody radioimmunoassay was developed.

The MM immunoassay showed no cross-reactivity with BB enzyme and only 15% cross-reactivity with the MB hydrid. In 50 healthy people enzyme activity has a 0.94 correlation coefficient with the radioimmunoassay determination, with normal values of 33 to 528 ng per ml of serum.

The BB immunoassay showed no cross-reactivity for MM enzyme and 5-15% cross-reactivity with MB. Minimal detectable amounts were 1.2 ng/ml of serum (in 100 ml). This allowed the measurement of BB in healthy people and patients since normal levels appear to be 2 to 11 ng/ml.

Significance to Biomedical Research: Measurement of creatine kinase serum enzyme molecules rather than catalytic activity should provide more useful clinical information about tissue damage in a variety of contexts. The radioimmunoassay's sensitivity and ability to distinguish among creatine kinase

isoenzymes should offer new diagnostic potentials. These methods should be applicable to other human enzymes now routinely measured.

Proposed Course of Project: These radioimmunoassays will continue to be used in parallel to standard enzyme assays in the Clinical Pathology Department to assess the utility of this new method. The radioimmunoassay will be applied in other clinical situations, such as tissue culture means of detecting muscular dystrophy heterozygotes. Further effort to obtain an assay specific for the hybrid MB enzyme will be continued. Development of radioimmunoassays for other clinically significant isoenzyme systems, such as aspartate amino transferase, will be initiated.

| SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space) | U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT | PROJECT NUMBER Z01 AM 25,021-02 LCB |
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PERIOD COVERED
July 1, 1976 to September 30, 1977

TITLE OF PROJECT (80 characters or less)
Studies of the Kinetics of Gelation of Sickle Cell Hemoglobin: Peptides as Possible Inhibitors

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

| | | | |
|---|---|---|---|
| PI: | C.T. Noguchi | Guest Worker | LCB NIAMDD |
| | A.N. Schechter | Chief, Section on Macromolecular Biology | LCB NIAMDD |

COOPERATING UNITS (if any)

LAB/BRANCH
Laboratory of Chemical Biology

SECTION
Macromolecular Biology Section

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20014

| TOTAL MANYEARS: | PROFESSIONAL: | OTHER: |
|---|---|---|
| 1.2 | 1.2 | 0 |

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

To investigate the antisickling potential of various agents on deoxygenated hemoglobin S, we have used the assay developed by Hofrichter, Ross and Eaton which monitors gelation kinetics via turbidity changes at 800 nm and measures solubility using ultracentrifuge sedimentation. The antisickling effect as measured by this assay has been determined for the amino acids, some dipeptides and several aromatic compounds. The aromatic amino acids, and phenylalanine analogs have been found to inhibit the gelation of deoxyhemoglobin S and hence may indicate a potential class of therapeutic anti-sickling agents. This project will be expanded to other compounds including oligopeptides in a continuing effort to study the surface interactions of deoxyhemoglobin S.

Project Description:

Objectives: The study of the gelation process of sickle cell hemoglobin has been of continuing interest to this laboratory. We have focused on the kinetics of gel formation and the solubility of sickle cell hemoglobin. Studies on nuclease and other proteins demonstrating the complementing effects of protein fragments have suggested to us that hemoglobin fragments may affect the gelation of sickle cell hemoglobin. If gel formation can be sufficiently retarded, it is believed that the tendency of a sickle-red blood cell to deform or sickle can be reduced or eliminated. The goal of this project is to study the effects of amino acids, dipeptides, oligopeptides (chosen from the normal and sickle hemoglobin sequence) and other compounds on the kinetics and solubility of sickle hemoglobin.

Methods Employed: Large amounts of hemoglobin S have been prepared by ion exchange chromatography from units of SS and SA blood. The method of optically monitoring turbidity and ultracentrifugation developed by Hofrichter, Ross and Eaton has been adopted. In this assay, concentrated hemoglobin S (close to the concentration found in red blood cells) is deoxygenated with sodium dithionite in a tube sealed under nitrogen. The kinetics of gelation are monitored by observing turbidity changes at 800 nm at a controlled temperature - a delay period in gelation is usually observed and measured. The sample is then spun in a thermally controlled ultracentrifuge at high speed which collapses the gel into a pellet leaving a solution saturated with tetrameric hemoglobin. The hemoglobin saturation at that temperature is obtained from measurements on a Cary 17 of the supernatant concentration.

Fragments of the amino terminal end of beta S hemoglobin and several other sites of the beta hemoglobin chain believed to participate in gel formation as suggested by recent X-ray crystallography data have been synthesized by a modification of the Merrifield method. However, at these high concentrations of hemoglobin additives may have complex effects due to non-ideality. Thus we have begun these studies with simple amino acids and simple aromatic compounds to analyze these effects.

Major Results: The essential amino acids have been tested in the gelation assay. Most of the amino acids exhibited little or no effect on gelation or solubility of deoxyhemoglobin S. Only the aromatic amino acid, (phenylalanine, tryptophan and to some extent tyrosine) significantly affected gelation kinetics and solubility. Both the d and Leisomers of these aromatic amino acids gave similar results.

Phenol, aniline, salicylic acid and acetylsalicylic acid were assayed for their antisickling potential. These compounds enhanced, rather than inhibited, gelation indicating that aromaticity was not a sufficient condition for an inhibitory agent. Several phenylalanine analogs (hydrocinnamic acid, phenethylamine, benzylamine, and amphetamine) were also tested. These compounds behaved similarly to phenylalanine in inhibiting gelation, suggesting a class of compounds which may be of therapeutic value.

The phenylalanine results increase the significance of the recent X-ray crystalography data for deoxyhemoglobin S which indicate that $\beta^{85phe}$ is in intermolecular contact with $\beta^{6\ val}$, the sickle hemoglobin mutation. The

dipeptides, thr-phe and phe-ala, from the $\beta^{85}$ region were tested and the results were similar to those of phenylalanine alone. To further investigate binding at this region, oligopeptides from the $\beta^{85}$ region have been synthesized and will be assayed for their inhibitory effect on gelation.

Significance to Biomedical Research: Peptide-protein interactions can be used as a probe to study the surface structure of normal and mutant hemoglobins. Of particular interest is hemoglobin S which aggregates to form a viscous gel under deoxygenated conditions. Increasing the delay time of gelation and the solubility of hemoglobin S are the minimal criteria for a suitable drug in sickle cell anemia therapy. So far, we have found that some amino acids and some aromatic compounds have the anti-sickling tendency. By studying peptides from the hemoglobin sequence, we hope to increase the specificity of anti-sickling agents and we hope to obtain information about hemoglobin-hemoglobin interactions, which might prove useful in studying other hemoglobinopathies besides sickle-cell anemia.

Proposed Course of Project: The gelation and solubility studies on hemoglobin S will continue. The peptides from the intermolecular contact regions which have been synthesized will be tested as possible stereospecific inhibitors of gelation. The initial data on the amino acids and other aromatic compounds will be used as a reference with which data obtained from other additives such as oligopeptides will be compared. When indicated, studies of erythrocyte permeability and physiology (oxygen affinity, Bohr effect, etc.) will be done with selected peptides. The ultimate goal of the project will be to find an anti-sickling agent with high specificity which can effectively penetrate the erythrocyte but with minimal physiological and toxicological side effects.

Publication:

Noguchi, C.T. and Schechter, A.N.: Effects of amino acids on gelation kinetics and solubility of sickle hemoglobin. Bioch. Biophys. Res. Com. 74, 637-642 (1977)

| SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space) | U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT | PROJECT NUMBER Z01 AM 25,022-01 LCB |
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PERIOD COVERED
July 1, 1976 to September 30, 1977

TITLE OF PROJECT (80 characters or less)
Interaction of Complement Proteins with Leukocytes, Erythrocytes, and Antibodies

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

| | | | |
|---|---|---|---|
| PI: | B.F. Tack | Staff Fellow | LCB NIAMDD |
| | P.E. Lorenz | Guest Worker | LCB NIAMDD |
| | A.N. Schechter | Chief, Section on Macromolecular Biology | LCB NIAMDD |

COOPERATING UNITS (if any)

LAB/BRANCH
Laboratory of Chemical Biology

SECTION
Macromolecular Biology Section

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20014

| TOTAL MANYEARS: | PROFESSIONAL: | OTHER: |
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| 1.8 | 1.8 | 0 |

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The ability of phagocytic and lymphoid cells, including the macrophage, polymorphonuclear leukocyte (PMN), and B lymphocyte, to recognize and bind to immune complexes (IC) has been attributed to the interaction between an IC bound form of the third component of complement (C3) and cell surface receptors specifi for this protein. We are attempting to measure directly the equilibrium and kinetic parameters of the binding between activation products of human C3 and their receptors on human blood cells. The activation fragments C3b, C3c, and C3d have been generated by digestion with porcine elastase and subsequently purified. Each fragment has been radiolabeled by reductive methylation using formaldehyde and ($^3$H) sodium borohydride. The interaction with erythrocytes and different leukycyte populations will be assessed using a direct binding assay where equilib rium saturation, time course, and competition experiments are expected to charac terize the physio-chemical nature of these interactions. In addition, specific antibodies have been raised in sheep and rabbits to each of these fragments and will be used for (a) probes to identify receptor binding sites in C3b and (b) developing specific radioimmunoassays to assess in vivo complement activation.

Project Description:

Introduction: It has been shown that surface receptors specific for activation fragments of C3 are present on blood and tissue cells of many vertebrate species. C3 receptors have been detected using a method of assay where a purified leukocyte population is mixed with an indicator cell, usually a sensitized sheep erythrocyte coated with an activated fragment of C3, and the cell suspension scored for rosetting of the indicator cell about the test cell after an appropriate incubation period. With this assay two distinct C3 receptors have been recognized: (1) the C3b receptor (CR1) present on the PMN, macrophage, B lymphocyte, and primate erythrocyte and (2) the C3d receptor (CR2) present on the macrophage and B lymphocyte. Many laboratories have contributed to our present knowledge concerning the specificity and biological manifestations of these interactions and the following observations are considered of particular significance: (1) CR1 and CR2 bind to topographically distinct regions of the C3 molecule (i.e. CR1 binds to the C3c domain of C3b and CR2 binds to C3d and to a lesser extent with the C3d domain of C3b), (2) the primary role of 1C bound C3 is to facilitate contact with phagocytic cells; although the presence of C3 is a necessary condition it is not alone a sufficient condition for the uptake of bound 1C by these cells, (3) both receptors are able to function quite independently in establishing contact between circulating 1C and the macrophage, (4) there is an obligatory C3 requirement for complement receptor positive B lymphocytes to respond in vivo and in vitro to T cell dependent antigens suggesting that CR1 and/or CR2 are required for T cell-B cell cooperative responses, perhaps at the macrophage level, (5) specific antibody inhibition studies have indicated that C3 receptors present on human lymphoid cells are identical to the HLA antigens 4a/4b (W4/W6), and (6) a close association has been observed between C3 receptors on human lymphoid cells and receptors for the Epstein-Barr virus. Therefore, in addition to their specific involvement in immune clearance pathways these receptors might well be linked to genetic mechanisms serving to modulate B lymphocyte activities.

Objectives: We propose to study in a quantitative manner the binding of activation products of human C3 to their respective surface receptors, CR1 and CR2, on human leukocytes. These studies will involve (1) the generation and subsequent purification of C3b, C3c, and C3d from limit proteolytic digests of the precursor protein, (2) the development of a direct binding assay that will allow kinetic and equilibrium parameters of the binding of these fragments to leukocytes to be assessed, (3) the inhibition of C3b, C3c, and C3d binding by competition with native or synthetic peptides from their respective structures, and (4) the isolation of antibodies to defined regions of the C3 molecule from native antisera using isolated activation fragments bound to solid matrices as specific immunoadsorbents. In addition, antibodies will be produced to each of the major activation fragments by direct immunization of sheep and rabbits. These reagents will be used for (a) probes for localization of receptor binding sites in C3b by specific antibody inhibition assays, (b) studies of the distribution of fragments on cell surfaces with fluorescein and ferritin labels, and (c) developing specific radioimmunoassays for each of the major activation peptides. We expect that data obtained from the above

physio-chemical and immunological approaches will contribute to our understanding of the topology of functional binding sites in the C3 protein and the basis for recognition between these receptors and their respective polypeptide ligands.

Results: Optimal conditions for the generation of C3 activation products, including C3a(MW 9200), C3b(MW 179,000), C3c(MW 147,000), and C3d(MW 32,000), by digestion of the native protein with porcine elastase have been established. This enzyme was chosen on the basis of preliminary analytical gel experiments which indicated that the activation products generated with elastase were indeed similiar in their molecular weights and immunoelectrophoretic mobilities to the "physiological" C3 fragments that have been purified, albeit in poor yield, from activated serum. Although we have used porcine elastase for these studies, experiments in our laboratory have indicated that identical cleavage products are obtained with human leukocyte elastase. The similarities between in vivo and in vitro cleavage patterns may not be fortuitous since elastase activity has been detected in plasma and elastase is a recognized constituent of PMN lyzosomes that has been implicated as one causative agent of tissue injury associated with inflammatory reactions.

Digestion of C3 (2-4 mg/ml; pH 8.2) with 1% w/w elastase for 2 min. at 37°C effects a specific and quantitative cleavage to C3a and C3b. Once elastase has been irreversibly inactivated with phenylmethylsulfonyl fluoride and the pH lowered to 5.6 these two activation products are resolved on a G-100 column equilibrated with 50 mM sodium acetate buffer (pH 5.6), 1 M in NaCl. Digestion for an additional 240 min. resulted in the conversion of C3b to C3c and C3d which are also separated on the above G-100 column.

In consideration of our proposed cell binding studies it has been necessary to radiolabel C3 and each of the purified activation products. Initially, we iodoinated C3 and C3b with carrier free $Na^{125}I$ and chloramine T. However several practical problems were encountered, including a high degree of non-specific binding to human red blood cells and a possible label induced loss of cell binding activity, with this labeling technique. Therefore we have adopted a method of labeling with tritium that promises to overcome the above objections. Specifically, the epsilon amino groups of the ligand are reacted with formaldehyde (pH 8.9) in the presence of tritiated sodium borohydride (specific activity: 10 Ci/mmole) to form the stable N-methyl and N, N-dimethyl derivatives of lysine residues. Labeling experiments with C3 have been encouraging as we obtained a tritiated protein with a specific activity of $5 \times 10^5$ cpm/ug which appears to have good human red blood cell binding activity. Inhibition experiments with unlabeled C3 have indicated that the binding of labeled material can be stoiciometrically inhibited. Therefore, methylation of lysine residues appears not to perturb the receptor binding sites of this protein.

In order to obtain quantitative information concerning the equilibrium and kinetic parameters of interactions between these polypeptide ligands and their cell surface receptors we require a direct binding assay which permits a rapid and complete separation of free and cell bound ligand. The method of assay that we are presently evaluating is carried out in the following manner: (a) the tritiated ligand is incubated with the test cell in isotonic Tris-HCl buffer (pH 7.4) containing 1% bovine serum albumin at 37°C with periodic agi-

tation, (b) an aliquot of the cell suspension is then layered on a liquid column (1.4:1 v/v mixture of dibutyl phthalate and bis-(2-ethylhexyl) phthalate) contained in a 400 microliter Beckman Microfuge tube and the cells rapidly pelleted thru the oil into a 20 microliter volume of 2% sodium dodecyl sulphate (40% in glycerol) present in the tip of the tube, and (c) the tip of the tube is then removed with a razor blade and the contents solubilized in scintillation fluid and counted. With this assay we have established that binding between human red blood cells and C3 is a saturable reaction that can be stoichiomertically inhibited with unlabeled C3. Further binding studies with C3 and each of the purified activation fragments, including equilibrium saturation, time course, and competition experiments, are expected to characterize the physio-chemical nature of these interactions.

Antibodies to C3 and each of the activation products have been successfully raised in both sheep and rabbits. We are now involved with the development of radioimmunoassays for each of these proteins. In addition, these fragments are being coupled to Sepharose 4B and the resultant conjugate used as a specific immunoadsorbent with which to obtain specific antibodies from antiserum made to the native protein. The tritiated ligands for cell binding studies are, as well, being used in these antibody assays.

Significance to Biomedical Research: One model for the pathogenesis of autoimmune disease (AID) would involve the fixation of humoral antibodies at specific tissue sites. Subsequent activation of the complement system at extravascular sites of antigen-antibody interaction would then effect the adherence of mobilized neutrophils to immune complex bound C3b and/or C3d and the release of lysomal hydrolases responsible for tissue injury. The participation of specific complement proteins in the pathogenic mechanisms of AID is presently an area of intensive investigation. A representative list of disease states in which humoral antibodies are believed to be elicted to specific tissue or cellular elements would include systemic lupus erythrematosus, rheumatoid arthritis, acquired hemolytic anemia, and glomerulonephritis. In vivo complement fixation on the glomerular basement membrane has been observed in lupus erythrmatosus and glomerulonephritis through fluorescent antibody studies of tissue obtained by renal biopsy. Further, the synovial tissue of patients with rheumatoid arthritis has reduced levels of several complement proteins that may be attributable to complement fixation by rheumatoid factor: gamma globulin complexes; finally, the red blood cells from patients with cold-agglutinin hemolytic anemia are found to be coated with C3 which targets these cells for sequestration by the reticuloendothelial system.

Elucidation of the molecular modes of interaction for these membrane oriented complement functions will require (1) further characterization of the structures of the individual complement proteins involved and (2) an evaluation of the physio-chemical basis for their binding properties. We intend to meet both of these requirements in our studies. The isolation of major activation fragments and subsequent chemical analysis will contribute to further structural delineation of the C3 molecule. Once the major cleavage fragments of C3 with ligand activities have been identified using the direct binding assay, studies can be initiated to determine the kinetic and equilibrium parameters of the interaction. Specific peptide and antibody inhibition experiments may establish subregions of these molecules that constitute receptor

binding sites. If such oligopeptides can be identified using these techniques, they may have specific application as inhibitors of complement binding. Thus this approach has the potential of offering a new class of inhibitors to the processes involved in the inflammatory reaction, especially in the class of autoimmune phenomena.

The development of specific radioimmunoassays for the activation products of C3 is expected to allow more precise measurement of (1) the extent of involvement and (2) the degree of activation of this protein in certain immune and autoimmune disorders.

| SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space) | U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT** | PROJECT NUMBER ZO1 AM 25,023-01 LCB |
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PERIOD COVERED

July 1, 1976 to September 30, 1977

TITLE OF PROJECT (80 characters or less)

Isolation and Characterization of the Mating Substances (M-agglutinins)

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

| | | | |
|---|---|---|---|
| PI: | Edward Steers, Jr. | Research Biologist | LCB NIAMDD |
| | Audrey Barnett | Guest Worker | LCB NIAMDD |
| OTHER: | Clifford E. Lee | Physical Science Technician | LCB NIAMDD |

COOPERATING UNITS (if any)

LAB/BRANCH

Laboratory of Chemical Biology

SECTION

Protein Chemistry Section

INSTITUTE AND LOCATION

NIAMDD:NIH; Bethesda, Md. 20014

| TOTAL MANYEARS: | PROFESSIONAL: | OTHER: |
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| 1.5 | 1 | 0.5 |

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Mating (or conjugation) in Paramecium occurs between complementary cells during special periods of reactivity. The interaction between reactive cells shows a high degree of specificity. When cells of opposite mating type are mixed under the appropriate conditions, they agglutinate into clumps containing from two to twenty animals. The agglutination phase is followed by a separation of the clumped cells into complementary pairs. Following a reciprocal exchange of gametic nuclei, the cells separate and undergo mitotic division.

The agglutinating reaction of paramecium, which triggers the cellular events leading to nuclear exchange and cell division, is reminiscent of another class of agglutinins - the lectins. The project outlined here is directed toward the isolation and characterization of the mating substances (M-agglutinin) of paramecium. Such characterization should lead to an understanding of the molecular basis for the specificity of agglutination in the mating reaction as a model for describing this specific cell-cell interaction.



PHS-6040
(Rev. 10-76)

Project Description:

Objective: To purify and characterize the mating substance(s) (M-agglutinins) from Paramecium multimicronucleatum. To characterize on a chemical level the basis of the specific interaction between complementary M-agglutinins.

Methods Employed: Culture methods have been adapted to grow large (10 to 20 liter) cultures of P. multimicronucleatum in both the reactive and unreactive state. Several procedures have been tested for isolating cilia free of other cellular debris. Antisera is being prepared against both unreactive and reactive cilia of mating types III and IV as well as against brei's of whole cells of both types. Cells harvested from 10 to 20 liter lots, yielding 1 to 2 ml of packed cells, are homogenized in the appropriate buffer and the resulting extract fractionated by various methods including ammonium sulfate precipitation, gel filtration and ion-exchange chromatography. Agglutinability of fractions will be assayed using reactive cilia from complementary types and homologous antisera. Inhibition, as well as induction, of agglutination is carried out in depression slides and monitored microscopically.

Major Findings: The initial agglutination reaction appears to involve only the cilia of the animals and only those cilia located on the anterioventral surface. The agglutination is a loose one which can be broken up or dissociated by mild agitation. The initial agglutination reaction is followed after one hour by membrane fusion and is characterized by pairs of cells which are tightly held together along the entire ventral or oral surface. Involvement of the cilia in agglutination has resulted in isolating reactive cilia which agglutinate with reactive cilia of the opposite mating type or with reactive cells of the opposite type. Experiments are now in progress to develope immune sera to reactive cells and reactive cilia for assay purposes in monitoring the purification of the mating substance(s). Two types of experiments suggest that the mating substance is proteinaceous; reducing concentrations of dithiothreitol show a significant inhibition of the mating reaction and prevent subsequent pair formation without being lethal to the cells, and preincubation of reactive cells with trypsin and pronase inhibits the mating reaction.

The recent studies on the ability of plant lectins to agglutinate various animal cells suggest a similar phenomenon may exist in paramecium. Several lectins and sugars are being tested for their effect on agglutination and pairing in paramecium in an effort to develope an alternate assay and gain insight into the molecular mechanisms of the specific cell- cell interactions associated with mating.

Significance to Biomedical Research: Mating in paramecium is characterized by a highly specific interaction between cells of opposite mating types. Specific cell- cell interaction is a universal phenomenon which is important in understanding various aspects of cell development. Knowledge of the molecular basis for the specificity of the mating reaction is important in understanding various types of cell interactions as well as understanding the architecture of cell surfaces associated with such reactions.

Proposed Course: The principal objective of this project (purification and characterization of the Mating substance) requires an assay to monitor the various steps employed. The agglutinability of reactive cilia is intended to serve as such an assay. The development of an immunological assay offers certain advantages, however, in following protein purification. With the development of an appropriate assay, purification of the mating substance will be undertaken using both the standard methods of purification as well as immunoaffinity chromatography. Characterization of the mating substances will include a determination of molecular weight, possible subunit structure, chemical composition, and identification of reactive groups. Subsequent studies will include attempts to follow the synthesis of mating substances relative to the cell cycle and those factors which induce synthesis and trigger mitosis resulting from cell - cell interaction. Localization of the mating substances on the cell (cilia) surface will also be attempted.

Publications:

None

| SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space) | U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT | PROJECT NUMBER Z01 AM 25,024-01 LCB |
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PERIOD COVERED
July 1, 1976 to September 30, 1977

TITLE OF PROJECT (80 characters or less)
Physical Chemical Studies of Self Assembling Protein Systems: Hemoglobin S and Tubulin

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

| | | | |
|---|---|---|---|
| PI: | J.W.H. Sutherland | Visiting Fellow | LCB NIAMDD |
| | A.N. Schechter | Chief, Section on Macromolecular Biology | LCB NIAMDD |

COOPERATING UNITS (if any)
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D. Torchia (Laboratory of Biochemistry, NIDR)

LAB/BRANCH
Laboratory of Chemical Biology

SECTION
Macromolecular Biology Section

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20014

| TOTAL MANYEARS: | PROFESSIONAL: | OTHER: |
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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The rotational motion of sickle hemoglobin in gels has been monitored by $^{13}C$ NMR with new decoupling procedures as a probe of the gelation process upon deoxygenation. It has been possible to quantitate the degree of immobilization in gells from cell-free hemoglobin solutions, as well as the intracellular gelation or sickling process. In addition, theoretical analysis of the results of recent inhibition studies of the gelation by amino acids indicate that the inhibition may be due to direct, but very weak, binding between amino acids and hemoglobin.

Detailed theoretical analyses of the disagreement between calorimetric and van't Hoff enthalpies of tubulin polymerization cast doubt upon the interpretation of many in vivo microtubule assembly studies. These analyses may be extended to include kinetic aspects of sickle hemoglobin gelation.



040
10-7

Project Description:

Objectives: Hemoglobin S and tubulin are examples of a variety of biologically important self-assembling systems which have been the focus of intense scrutiny recently: hemoglobin S, because of its importance in sickle cell disease; and tubulin, because microtubules have been implicated in some processes of cellular transport and cyto-skeletal support. This report outlines some thermodynamic and kinetic considerations arising in connection with each protein system.

Hemoglobin gelation: In sickle-cell disease, the presence of a mutation in the β polypeptide chain of the mutated hemoglobin causes solutions of this hemoglobin (hemoglobin S) to gel under appropriate conditions. A recent assay developed by Eaton, Hofrichter and Ross of NIAMDD has made possible the quantitative study of gelation in cell free hemoglobin S solutions. We are studying: (i) gelation in intact cells, where this standard assay is inapplicable; and (ii) the interpretation of recent amino acid inhibition studies by Noguchi and Schechter (BBRC 74, 637-642, 1977) as to the mode of inhibition, an important consideration in the design of any screening program for inhibitors.

Tubulin: A detailed theoretical analysis of the disagreement between calorimetric and van't Hoff enthalpies of assembly has been completed.

Methods: Hemoglobin Gelation: (i) The comparison of the natural abundance $^{13}C$ NMR spectra of sickle hemoglobin with magnetic dipolar decoupling and cross-polarization techniques affords a direct measure of the fraction of immobilized hemoglobin in any sample. The technique is totally independent of spectral assignments and is applicable to whole cells or to chromatographed cell-free hemoglobin. (ii) Theoretical analyses of the gelation mechanism are based upon nucleation theory. Tubulin: This analysis is also based upon nucleation theory.

Major Findings: Hemoglobin Gelation: (i) Our preliminary studies indicate that about 25±5% of the hemoglobin in a purified gel is immobilized. Gelled cellular hemoglobin S appears much more mobile. The rotational correlation time of unpolymerized hemoglobin appears to be totally unaffected by the presence - or absence - of microtubular polymers. (ii) Analysis of the Noguchi data show that aromatic amino acids could be inhibiting gelation by direct binding to hemoglobin, albeit with a small binding constant ($\sim 3\ M^{-1}$). Excluded volume effects must be rejected as a possible explanation of inhibition and are predicted, in fact, to enhance gelation. The possible influence of amino acids upon non-ideality effects in hemoglobin solutions (with corresponding effects on hemoglobin solubilities) is still unknown. Tubulin: The enthalpy of polymerization is an important parameter because it provides a quantitative measure of the effect of temperature upon the extent of microtubule assembly. this, in turn, is of interest because the extent of microtubule assembly has been correlated with the movements of various cell organelles (e.g. The polymerization-depolymerization theory of chromosomal movement). Previous reports based upon the effect of temperature upon microtubule assembly in whole cells have indicated that the enthalpy of tubulin polymerization may be as large as 80 kcal/mole of tubulin, yet direct calorimetric measurements by

Sutherland and Sturtevant (PNAS 73, 3565, Oct. 1976) show that the actual enthalpy change is only $\sim 0 \pm 1$ kcal/mole. This discrepancy has been resolved by a theoretical analysis. The studies performed on whole cells yield an indirect "van't Hoff" enthalpy of polymerization, which is a complex function of the individual equilibrium constants for the various steps in the overall assembly mechanism. It has also been found that the tubulin polymerization-depolymerization theory of chromosomal movement violates certain fundamental physical principles.

Proposed Course of Research: Hemoglobin S Gelation: (i) The surprising preliminary indication that gelled cellular hemoglobin is more mobile than chromatographed cell-free hemoglobin will be investigated further. This will entail quantitative comparison of spectra of SS and SA cells, of packed pellets containing nearly 50% of polymerized hemoglobin, and of crystals of hemoglobin S which have recently been suggested by Steinhart as an alternative to gels as a cause of cellular sickling. (ii) Kinetic studies of the gelation process will be undertaken by monitoring changes in the rotational motion of 2,3-diphosphoglycerate bound to sickle hemoglobin. The requisite $^{31}P$ spectra can be obtained much more rapidly than with $^{13}C$ NMR because $^{31}P$ is the naturally occurring phosphorus isotope. All available physical chemical data on the binding of 2,3-diphosphoglycerate to hemoglobin (e.g., Benesch and Benesch [1969, 1970] and Chance [1970]) indicate that the binding is stoichiometric, tight ($K_b \sim 3 \times 10\ M^{-1}$) and rapid ($k_f \sim 10^{-3}\ M^{-1}\ sec^{-1}$, $k_b \sim 10^{-7}\ sec^{-1}$). Moreover, for technical reasons, the decoupling procedures should be simpler than for $^{13}C$ NMR. All experiments performed to date by $^{13}C$ NMR on cell free hemoglobin can thus be confirmed by $^{31}P$ NMR, and additional kinetic information obtained. (iii) The possibility of direct interactions between deuterated aromatic amino acids and hemoglobin will be studied by deuterium NMR. This investigation will monitor changes in the linewidth of the $^{2}H$ label and will attempt to determine the binding constant of the amino acid to the hemoglobin.

Significance to Biomedical Research: Hemoglobin Gelation: (i) Our $^{13}C$ NMR techniques may well result in the first quantitative assay of hemoglobin gelation in whole cells; (ii) $^{31}P$ NMR may lead to a new kinetic probe of hemoglobin gelation which might be sensitive to clinically important pregelation phenomena which are, at present, undetectable; and (iii) $^{2}H$ NMR may confirm theoretical analyses of the inhibition of hemoglobin gelation by amino acids. Tubulin: Chromosomal movement has been ascribed to the polymerization-depolymerization of tubulin into microtubular polymers present in spindle fibers. This process has been analyzed thermodynamically in whole cells using temperature as the experimental variable. The new results indicate the need for a reassessment of all such *in vivo* studies performed to date and cast doubt upon the polymerization-depolymerization theory of chromosomal movement.

Publication:

Sutherland, J.W.H.: Disagreement Between Calorimetric and van't Hoff Enthalpies of Assembly of Protein Supramolecular Structures. Proc. Natl. Acad. Sci. 74: 2002-2006, 1977.

| SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space) | U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT** | PROJECT NUMBER Z01 AM 25,025-01 LCB |
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PERIOD COVERED
July 1, 1976 to September 30, 1977

TITLE OF PROJECT (80 characters or less)

Studies on the Structure-Function of Cytochrome c Using a Fragment Complementing System

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

| | | | |
|---|---|---|---|
| PI: | Gary R. Parr | Staff Fellow | LCB NIAMDD |
| | H. Taniuchi | Chief, Section on Protein Conformation | LCB NIAMDD |

COOPERATING UNITS (if any)

LAB/BRANCH
Laboratory of Chemical Biology

SECTION
Protein Conformation Section

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20014

| TOTAL MANYEARS: | PROFESSIONAL: | OTHER: |
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CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

A heme containing fragment of cytochrome c consisting of amino acid residues 1 through 38 (1-38H) was prepared by tryptic digestion of the native molecule. Apocytochrome c (1-104) has also been prepared by removal of the heme moiety through standard chemical techniques. On mixing these two fragments a non-covalently linked 1:1 complex is formed, and the spectral properties and biological activity of this complex are essentially identical to native cytochrome c. The redundant residues of this native-like complex were removed by tryptic digestion of the material in its reduced form ($Fe^{+2}$). Subsequent separation and identification of the remaining complimenting fragments revealed that two distinct complexes are formed from the initial mixture of (1-38H) and apoprotein. Thus one complex consists of residues (1-25H) from the original (1-38H) fragment and residues (23-104) from the apoprotein, while a second complex utilizes the entire (1-38H) fragment and residues (56-104) from the apoprotein. In addition, we have shown that these native-like species may be regenerated by mixing the appropriate non-overlapping fragments. Biologically active complexes have been prepared from mixtures of (1-25H) + (23-104) and (1-38H) + (56-104) as well as (1-38H) + (39-104).

PHS-6040

Project Description:

Methods Employed and Major Findings: A heme containing fragment of cytochrome c, (1-38H), and the apoprotein, (1-104), were prepared and purified by standard techniques. Complimentation of these disordered fragments was monitored by observing the quenching of the fluorescence of the single tryptophan residue at position 59 in the amino acid chain. When (1-38H) is added to a solution of (1-104) a linear decrease in fluorescence intensity is observed until the concentrations of the fragments are equal. Excess (1-38H) produces no additional decrease in fluorescence intensity. This experiment indicates that a tightly bound, 1:1 complex of the fragments is formed.

The biological activity of the complexes was determined with a lactate dehydrogenase (cytochrome b2) assay system. The activity of an equimolar mixture of (1-38H) and (1-104) was found to be identical to that of native cytochrome c at the same concentration. Furthermore, a plot of relative activity vs. the ratio of (1-104):(1-38H) shows a linear increase when the ratio is less than one and no change with excess apoprotein. This confirms the result of the flourescence experiment and, in addition, suggests that the non-covalent complex formed by the overlapping fragments is a highly ordered structure whose conformation must closely resemble that of native cytochrome c. This conclusion is reinforced by the similarity of the spectral properties of the non-covalent complex and native cytochrome c, particularly the existence of the 695 nm band (indicative of ligation of met-80 with the iron atom) in the complex.

Since the native-like material formed from these fragments theoretically has more amino acid residues than are necessary to determine the three dimensional conformation, certain portions of one or both fragments should be redundant and thus not used to form the final structure. This was demonstrated by incubation of reduced complex with trypsin followed by gel filtration on Sephadex G-50 to isolate the remaining native-like material (recovery ~85% of theoretical), and subsequent separation and identification of the complementing fragments (see the report of the previous year from this section). The identity of the resulting fragments was determined by amino acid analysis, peptide mapping, and manual Edman degradation. Two heme containing peptides, (1-25H) and (1-38H), and two apopeptides, (23-104) and (56-104), were found. Fluorescence quenching and biological activity titration experiments (described above) indicated strong binding ($K_d < 10^{-7}$ M) and substantial activity for the combinations (1-25H) + (23-104) and (1-38H) + (56-104) but much weaker binding and no biological activity for the combination of (1-25H) + (56-104). These results, coupled with estimates of the relative amounts of each fragment indicate that two distinct complexes are formed and that under the conditions of the experiment the ratio of (1-25H) (23-104) to (1-38H) (56-104) is about two. The complex of (1-38H) (56-104) is of particular interest in that the missing residues correspond exactly to a region of the sequence of eukaryote cytochrome c which does not appear to exist in pseudomonas bacteria.

Additional work with other fragment combinations has shown that a viable native-like complex may be formed from a mixture of (1-38H) and (39-104), while a mixture of (1-25H) and (39-104) results in weak binding and no biological activity.

Significance to Biomedical Research: The understanding of interatomic interactions underlying the conformational dynamics and functions of proteins is important in understanding of cellular mechanisms involving proteins. This new fragment complementing system of cytochrome c developed here would be an excellent test object for examination of the contribution of individual amino acid residues to the interatomic interactions responsible for the structure and function of cytochrome c, an important class of proteins.

Proposed Course of Research:

1) Stopped-flow kinetic studies of the complementation reaction of various fragment combinations to determine the energy barrier of folding, and to look for differences in reaction rates which may help in understanding the mechanism of protein folding.

2) Determination of the redox potential of the iron atom in the various complementing pairs to aid in understanding the relationship between the redox potential and the conformational stability.

3) Further comparison of the properties of the native-like complexes through techniques such as circular dichroism and folding-unfolding transitions.

4) Preparation of the fragment (39-91) to determine the importance of the carboxy-terminal region in maintaining the native structure.

5) To conduct experiments to determine the factors governing the relative amounts of the two complexes formed from the initially redundant mixture of overlapping fragments.

6) Crystallization and X-ray diffraction studies of the complexes to compare these structures in a more detailed way to that of the native protein.

7) Preparation of inter-species complementing systems to determine the effect of specific sequence modifications.

8) Development of a sensitive stopped-flow light scattering system to compare rates of molecular weight changes with rates of spectral changes in complementation reactions.

# ANNUAL REPORT OF THE LABORATORY OF BIOCHEMICAL PHARMACOLOGY NATIONAL INSTITUTE OF ARTHRITIS, METABOLISM, AND DIGESTIVE DISEASES

## I. PROTEIN STRUCTURE AND MECHANISM OF ENZYME AND HORMONE ACTION

*Studies on Structure and Function of Thyrotropin Receptor*

The two components of thyroid plasma membranes (the glycoprotein with specific TSH binding activity, and the gangliosides of the thyroid membranes), known to interact with thyrotropin (TSH), have been segregated when membranes are solubilized with lithium diiodosalicylate and individually studied. Each component contributes to the properties of the intact plasma membrane receptor, and the functional transmission of the TSH message to the thyroid cell machinery requires the presence of both components.

Thyroid plasma membranes are not only rich in gangliosides with the chromatographic characteristics of $G_{D1b}$, $G_{T1}$, and $G_{M1}$, but also in higher order gangliosides which (i) are even more potent inhibitors of TSH binding and (ii) appear to be absent in the brain. Luteinizing hormone (LH) and human chorionic gonadotropin (hCG) have similar receptor structures with unique patterns of ganglioside inhibition.

TSH causes an increase in the uptake of the lipophilic cation triphenylmethylphosphonium ($TPMP^+$) when added to cultured thyroid cells or to membrane vesicles derived from these cells. This effect depends upon a specific interaction of TSH with its receptor at the cell surface. Since uptake of $TPMP^+$ and other lipophilic cations has been shown to reflect the presence of an electrochemical potential across the membrane, one of the primary effects of TSH is to hyperpolarize the thyroid cell membrane.

The effect of TSH on $TPMP^+$ uptake precedes the effect of the hormone on adenylate cyclase activity. Thus the possibility exists that a primary mode of action of each of these effectors is to alter electrochemical ion gradients across the cell membrane. In this regard, the sequence homology noted between the A protein of cholera toxin and $\alpha$ subunit of the glycoprotein hormones is shown to be startlingly similar in sequence to the nonapeptide neurohypophyseal hormones, oxytocin and vasopressin: nonapeptides, whose action also involves adenylate cyclase stimulation and altered ion flux across renal and muscle membranes.

. . . . . Drs. L. D. Kohn, S. M. Aloj, P. H. Fishman (Developmental and Metabolic Neurology Branch, National Institute of Neurological and Communicative Disorders and Stroke), E. F. Grollman, F. D. Ledley, G. Lee, M. F. Meldolesi, B. R. Mullin, and H. R. Kaback (The Roche Institute of Molecular Biology, Nutley, New Jersey)

*Studies on Relationships between Glycoprotein Hormones and Bacterial Toxins*

Tetanus toxin was shown to interact with thyroid plasma membranes. The properties of this interaction were shown to closely resemble the properties of the TSH interaction with TSH receptors on these membranes; the interaction of tetanus toxin with thyroid membranes exhibited several characteristics of its

interaction with neural tissue; tetanus toxin would not bind to membranes of a thyroid tumor with a TSH receptor defect; and tetanus toxin could stimulate thyroid hyperfunction in mice subjected to conditions known to precipitate thyroid storm.

The ability of tetanus toxin to interact with thyroid membranes and with TSH receptors on these membranes has an obvious clinical implication, i.e., the ability of the toxin to induce thyroid hyperfunction can contribute to the syndrome of sympathetic overactivity which can appear in tetanus. This syndrome would thus be analogous in its etiological relationship to the thyroid as is thyroid storm or as is the hypersensitivity to catecholamines demonstrable in thyrotoxic patients.

. . . . . Drs. L. D. Kohn, S. M. Aloj, W. H. Habig (The Bacterial Toxin Branch, Division of Bacterial Products, Bureau of Biologics, Food and Drug Administration), M. C. Hardegree (The Bacterial Toxin Branch, Division of Bacterial Products, Bureau of Biologics, Food and Drug Administration), F. D. Ledley, G. Lee, and M. F. Meldolesi

*Studies on Mechanism of Action of Interferon*

Evidence has accumulated that suggests the binding of interferon to specific cell surface receptors is necessary for the development of antiviral action and these receptors, like the glycoprotein receptors described above, contain gangliosides or ganglioside-like structures whose oligosaccharide moiety is a critical feature of the receptor structure.

Experiments using TSH or cholera toxin as probes of the mechanism of interferon action indicate the following: (i) The initial binding of interferon to a ganglioside or ganglioside-like receptor is insufficient to establish the antiviral state. (ii) The receptor for interferon has both a glycoprotein and a ganglioside as structural components. (iii) Immediately following the interaction of interferon with this receptor structure, there is a change in membrane state analogous to that seen when cholera toxin and TSH interact with thyroid membranes. (iv) The failure of human KB cells to transmit the interferon message to the cell machinery, despite the ability of interferon to bind to the cell surface of the cells, correlates with a near absence of membrane gangliosides, especially $G_{M2}$ and $G_{M3}$, which are present in high levels in membranes of sensitive mouse L cells. (v) Interferon stimulates adenylate cyclase activity in sensitive cells, maximal stimulation preceding the establishment of the antiviral state. (vi) Interferon induces changes in the electrochemical gradient across the cell membrane; this effect may be related to the ability of ouabain to inhibit interferon action.

. . . . . Drs. L. D. Kohn, R. M. Friedman (Laboratory of Experimental Pathology, National Institute of Arthritis, Metabolism, and Digestive Diseases), E. F. Grollman, G. Lee, and M. F. Meldolesi

*Studies on Structure and Function of Receptors for Other Glycoprotein Hormones*

Sequence homologies between LH, hCG, follicle-stimulating hormone (FSH), TSH, and cholera toxin raised the possibility that all glycoprotein hormones would

have a similar receptor structure and a similar mechanism of receptor interaction. Each target organ would thus have as its receptor a ganglioside or ganglioside-like structure with a specific and unique carbohydrate sequence, i.e., one which was different from that on other target organs. Ganglioside inhibition of hCG binding to testes membranes provides preliminary support for a portion of this hypothesis. The data show that the oligosaccharide moiety of the hCG receptor structure is distinct from the oligosaccharide moiety on the TSH receptor, i.e., the most effective inhibitors of hCG binding have a terminal sialic acid on the oligosaccharide chain, whereas the most effective inhibitors of TSH binding have a disialyl group linked to the internal galactose residue. Studies with LH are consistent with these conclusions since gangliosides also inhibit LH binding to testes membranes; the ganglioside specificity is distinct from that of both hCG and TSH; and the different gangliosides impose distinct conformational changes on hCG and LH which can be correlated with the inhibition data.

. . . . . Drs. L. D. Kohn, S. M. Aloj, and G. Lee

*Tryptophan Synthase of Escherichia coli*

The investigation of the effects of subunit interaction on the structure and function of the $\alpha$ and $\beta_2$ subunits of tryptophan synthase has been facilitated by the development of new methods for resolving the holo $\alpha_2\beta_2$ complex and for separating the $\alpha$ and apo $\beta_2$ subunits. The results show that pyridoxal 5'-phosphate is tightly bound in the holo $\alpha_2\beta_2$ complex and can only be removed after addition of a salt which causes subunit dissociation. The finding that pyridoxal 5'-phosphate derivatives are much more strongly bound to the $\alpha_2\beta_2$ complex than to the $\beta_2$ subunit indicates that strong binding forces, in addition to the Schiff base linkage, exist in the $\alpha_2\beta_2$ complex but not in the $\beta_2$ subunit.

The studies above suggest that pyridoxal 5'-phosphate may be bound at the subunit interaction site in the $\alpha_2\beta_2$ complex; this is consistent with chemical modification studies which show that certain active site residues (sulfhydryl, histidyl, and arginyl) appear to be buried in the subunit interaction site. These catalytically important amino acid residues are being located by peptide mapping studies. The location of an essential sulfhydryl residue is also being studied with a new affinity label, bromoacetyl pyridoxamine 5'-phosphate, synthesized by Dr. William Higgins.

. . . . . Drs. E. W. Miles and W. Higgins

*Aminoacyl tRNA Synthetases*

Over a period of years we have accumulated evidence that the glycyl tRNA synthetase of yeast can exist in two forms. These are present in the cells and vary in concentration in a reciprocal manner during growth. During the past two years we have sought to study the cyclic interconversion of the two forms in cell-free extracts with the objective of illucidating the detailed regulatory mechanisms. Recent findings indicate that at least two processes participate in causing changes thought up to now to be due to a single process.

. . . . . Dr. S. Black and Mrs. B. Hazel

## II. SYNTHESIS OF PROTEINS AND NUCLEIC ACID

*Nucleic Acid Synthesis*

Replication of linear duplex T4 DNA in vivo requires several proteins encoded by the phage DNA including T4 DNA polymerase, the T4 DNA unwinding protein (gene 32 protein), and the proteins encoded by genes 44, 62, 45, and 41. The complex of the gene 44 and 62 proteins is a DNA-dependent ATPase (or dATPase) whose activity is enhanced by the gene 45 protein. The 41 protein is a single-stranded DNA-requiring nucleotidase, which hydrolyzes both ribo- and deoxyribo-triphosphates.

The same six proteins are required for DNA synthesis in vitro with native duplex templates such as T4 DNA and the closed circular DNA from the plasmid col E1. Synthesis requires rATP or a high concentration of dATP and under optimal conditions leads to product in excess of the DNA used as template. By electron microscopic studies (in collaboration with Drs. Boris M. Peterlin and Jacob V. Maizel, National Institute of Child Health and Human Development), we find that a 3' OH at a nick in the duplex is used as a primer and that extension of this primer leads to displacement of the strand originally annealed to the strand serving as the template. At later stages, the product has double-stranded branches resulting from extensive synthesis of DNA complementary to the displaced strand. The unwinding of the template helix is closely coordinated with synthesis, since it does not occur in the absence of any of the proteins or nucleotides required for synthesis.

Under replicating conditions the 32 protein binds to supercoiled col E1 DNA at one of two specific areas, which we have mapped relative to the site of cleavage of this DNA by the ecoR1 restriction nuclease. The 32 protein sites correspond to those most easily denatured by alkali. By relating the length of the displaced strand to the R1 cleavage site or to denaturation bubbles, we have shown that synthesis on col E1 DNA with the T4 enzymes does not begin at a unique site.

. . . . . Drs. N. G. Nossal, B. M. Peterlin (National Institute of Child Health and Human Development), and J. V. Maizel (National Institute of Child Health and Human Development)

We have carried out a large scale search for temperature-sensitive mutants of E. coli which enhance the frequency of replication errors (mutator mutants), using a new method suitable for screening large numbers of clones in which $lac^+$ mutants arising in a $lac^-$ colony are identified as white papillae on the surface of red colonies growing on tetrazolium plates. Mutator strains are easily identified by this procedure, but only one of these mutators has proved to be temperature-sensitive. This mutation (mut3) appears to be in the structural gene (dnaE) for DNA polymerase III, the polymerase required for DNA replication, since it maps at the same position as previously isolated mutants in this gene and complements dnaE mutants in vivo. DNA polymerase III activity is not thermolabile in partially purified preparations from mut3, but the specific activity decreases after growth of the mutant at a nonpermissive temperature. The increased mutations (approximately 100-fold) are probably

due to the incorporation of incorrect nucleotides during DNA synthesis, since we have shown that they do not depend on the induction of the "error prone" repair system.

. . . . . Drs. N. G. Nossal and E. B. Konrad

*Protein Synthesis and Gene Expression in Escherichia coli*

We recently showed that although the concentration of the peptide chain elongation factor EF-Tu is maintained at an 8- to 14-fold molar excess above the concentration of ribosomes or EF-G or EF-Ts, the synthesis of EF-Tu is nonetheless coordinated with that of ribosomes EF-G and EF-Ts. We are now investigating two questions that arise from these findings: How does the cell synthesize so much EF-Tu? How does the cell coordinate the synthesis of EF-Tu with that of ribosomes and other macromolecules?

Pertaining to the first question, Nomura and coworkers recently showed that the cell contains at least two genes for EF-Tu (tufA and tufB), and work from this laboratory has recently shown that the EF-Tu's coded by these two genes are identical except for one peptide. Thus, unlike the only other example of a duplicated gene for a protein in E. coli, ornithine carbamyl transferase, the tufA and tufB genes have hardly diverged. This result suggests that the duplication of the tuf gene may serve only to maintain the high synthetic rate of EF-Tu. As was pointed out earlier, a high level of EF-Tu is consistent with its role in protein synthesis, which is to form a ternary complex with GTP and aminoacyl tRNA. It is in this form that aminoacyl tRNA gains access to the ribosome, and the amount of EF-Tu is approximately equimolar with that of aminoacyl tRNA at all growth rates. This fact prompted us to examine the relationship of EF-Tu and aminoacyl tRNA in crude extracts, and studies led us to the discovery of a new factor(s) in crude extracts which dissociates the aminoacyl tRNA from the ternary complex.

Our investigations of how the cell coordinates the synthesis of EF-Tu with the other components of the protein synthetic apparatus led us to the finding that like tRNA, rRNA, and ribosomal proteins, EF-Tu and EF-G are under stringent control, i.e., expression of the relA$^+$ allele causes a decrease in the synthesis of EF-Tu and EF-G relative to that of other proteins. We also showed the rather surprising result that the synthesis of a large number of proteins are influenced by the relA gene. We have continued these studies to show that during conditions which produce expression of the relA allele, derepression of the tryptophan biosynthetic pathway, but not the arginine biosynthetic pathway, is decreased in a relA$^-$ strain. In contrast, derepression of the arginine biosynthetic pathway, but not the tryptophan biosynthetic pathway, is decreased in an otherwise isogenic relA$^+$ strain. The latter finding suggests that the arginine biosynthetic enzyme is under stringent control, perhaps because this pathway provides the biosynthetic intermediates for the synthesis of polyamines which interact with many components of the protein synthetic apparatus.

. . . . . Dr. A. V. Furano

*The Mechanism of Action of Nerve Growth Factor (NGF)*

In collaboration with Dr. Lloyd Greene, we are studying the effect of the protein NGF on a line of cells cloned from a murine neural tumor. In the absence of NGF, the cells grow as small, round cells devoid of any of the histological and many of the biochemical features of neural cells. Upon the addition of NGF, essentially all of the cells stop dividing and after a period of differentiation assume most of the histological, physiological, and biochemical properties of a mature neural cell. We are currently analyzing to what extent the population of proteins synthesized by the differentiated cells differs from that synthesized by cells growing without NGF, at what time during differentiation the changes in protein synthesis occur, and by what means NGF produces these changes.

. . . . . Drs. A. V. Furano and Lloyd Greene (Department of Neuropathology, Children's Hospital Medical Center, Boston, Massachusetts)

## III. COMPLEX CARBOHYDRATES

A purified human monoclonal IgM(κ) (agglutinin MKV) has been characterized as both a cold agglutinin and a cryoglobulin. As its reactivity with human erythrocytes is reduced by treatment of the cells with ficin and its reactivity with both is abolished by treatment of the cells with neuraminidase, it has by definition blood group $Pr_2$ specificity. The membrane receptors for agglutinin MKV are sialic acid-containing glycoproteins in human cells and sialic acid-containing glycolipids in dog cells. Agglutination of erythrocytes is specifically inhibited by gangliosides $GM_3$ and N-acetylneuraminyl-paragloboside but not by their N-glycolylneuraminyl forms or by lactosyl ceramide or paragloboside, and the reactivity of neuraminidase-treated cells can be restored by allowing them to absorb either $GM_3$ or N-acetylneuraminyl-paragloboside. When large amounts of gangliosides are absorbed, the cells are agglutinated not only in the cold but also at 37°, showing that the density of receptor sites on the erythrocyte surface can influence the thermal amplitude of cold agglutinins and supporting our previous suggestion that cold agglutinins in general are simply low affinity antibodies which require multivalent attachment for the stabilization of bonds strong enough to agglutinate erythrocytes. Liberation of sialic acid from cold agglutinin MKV by treatment with neuraminidase does not affect its agglutinating properties, but the asialoprotein is no longer a cryoglobulin. Apparently the physical basis for its precipitation in the cold is the intermolecular immune binding of N-acetylneuraminyl residues.

Aminophenylethylamine derivatives of oligosaccharides conjugated to the hemp seed protein, edestin, are effective antigens. Rabbits immunized with the conjugates produce antibodies that bind radiolabeled oligosaccharides. Specificity of binding is determined by comparing inhibitory activities of structurally-related oligosaccharides in radioimmunoassays. All of three rabbits immunized with a mannotetraose derivative, two of five rabbits immunized with a lacto-N-fucopentaose III derivative, and four of five rabbits immunized with a lacto-N-tetraose derivative, developed useful

antibodies (i.e., antibodies with dissociation constants for the corresponding oligosaccharide of less than $10^{-5}$). These antibodies can be used to detect specific sugar sequences in complex carbohydrates.

. . . . . Drs. V. Ginsburg, D. A. Zopf, C.-M. Tsai, and D. F. Smith

## IV. THE OUTER MEMBRANE OF ESCHERICHIA COLI

The outer membrane is the outermost layer of the surface of E. coli. It is relatively simple in composition, is assembled from molecules of protein, lipid, and lipopolysaccharide (LPS) made elsewhere in the cell and therefore serves as a good model for studying membrane structure and assembly.

### *Assembly and Organization of the Escherichia coli Outer Membrane*

Newly synthesized LPS enters the outer membrane at approximately 10-50 insertion points and moves from these points elsewhere in the membrane, as shown by work in this and other laboratories. In current experiments we are isolating these insertion points and analyzing their composition to find how they differ from the remainder of the membrane.

. . . . . Drs. D. Filer and L. Leive

After an initial period when the LPS can move in the membrane, it appears to become immobilized into domains that do not mix with subsequently incorporated LPS, as shown by density labeling experiments. By immunological methods it has been possible to show that each domain may be approximately 0.1% to 1% of the cell surface in area, each containing approximately 1,000-10,000 LPS molecules. The nature of these domains are being further analyzed.

. . . . . Dr. L. Leive

Freeze-etch electron microscopy of cells treated with EDTA to remove half the LPS shows some changed and some unchanged areas, with respect to thickness and fracture properties. These areas form patches of about 1% of the cell surface. Such results also suggest heterogeneity of composition of different areas within the outer membrane.

. . . . . Drs. M. E. Bayer (Institute for Cancer Research, Philadelphia, Pennsylvania) and L. Leive

Several reports have suggested that lipid mobility in the outer membrane may be more restricted than in the cytoplasmic membrane of these and other cells. Using electron spin resonance spectral analysis, we found that outer membrane preparations containing LPS with a long polysaccharide chain were much less fluid than preparations containing LPS with a short polysaccharide chain, and treatment with EDTA to reduce LPS content increased mobility. These results indicate that the polysaccharide moiety of LPS restricts the mobility of the lipid hydrocarbon chains in the outer membrane.

. . . . . Drs. S. Rottem (The Hebrew University, Jerusalem, Israel) and L. Leive

*Permeability Function of the Membranes Studied with Mutants*

The outer membrane is known to be a molecular sieve which admits some molecules and excludes others. To study this barrier function further we have isolated novobiocin-supersensitive mutants. One class of mutant maps at minute 79 on the E. coli chromosome, shows increased permeability to a wide range of hydrophobic antibiotics, and apparently has some changes in composition of its LPS. Another, which maps at minute 11 on the E. coli chromosome, shows increased permeability to both hydrophobic and hydrophilic antibiotics and other substances, and has no obvious LPS changes. The protein and lipid composition of the outer membrane of these mutants, as well as a detailed study of the LPS, is continuing.

. . . . . Drs. W. G. Coleman, Jr., and L. Leive

*Physiology and Genetics of an Escherichia coli Strain Important in Studies of Outer Membrane*

E. coli O111 is of great interest both because it is a common E. coli strain enteropathogenic for infants and because it has a very well-characterized LPS and well-studied outer membrane. However, biochemical work has been hindered because standard genetic manipulations have been heretofore impossible in this strain. We have worked out procedures for conjugation and have derived substrains which can be transduced. Transductions of loci at different map positions indicate that the positions are the same as for E. coli K-12. The methods devised permit genetic analysis of this strain in as simple and complete a manner as E. coli K-12.

. . . . . Drs. W. G. Coleman, Jr., and L. Leive

## V. MECHANISMS OF INHERITANCE IN SACCHAROMYCES CEREVISIAE

*The "Killer Character" of Saccharomyces cerevisiae*

Killer strains of S. cerevisiae are those which harbor a $1.4 \times 10^6$ molecular weight double-stranded (ds) RNA encapsulated in a virus-like particle. This ds RNA (called the killer plasmid or killer virus) gives strains carrying it the ability to secrete a protein toxin which is lethal to strains not carrying this ds RNA species.

Since our last report, we have defined by mutation fourteen new chromosomal genes needed to maintain or replicate the ds RNA killer plasmid, called mak11 through mak24. (Mak stands for maintenance of killer.) Several of these mutations have been located on the genetic map of yeast: mak11, mak13, and mak15 are on chromosome XI, mak12 is on chromosome XII, and mak14 is on chromosome III.

A method has been devised to allow complementation tests between mak mutants. Improved methods for genetic mapping of general applicability for yeast are also being developed.

Mutations in two distinct mak genes are partially dominant to wild type.

Secondary mutations (suppressors) have been found which bypass various mak mutations. Absence of a nonprotein product of the mitochondrial DNA (perhaps a tRNA species) results in bypass of the mak10 gene. That is, the killer plasmid replicates in the absence of the mak10 gene product if (and only if) the mitochondrial DNA genome is defective for production of a particular nonprotein product.

A single dominant chromosomal mutation has been found which bypasses the killer plasmid's need for the mak1, mak4, mak5, mak6, and mak8 gene products.

Another dominant chromosomal mutation, at a different site, bypasses mak7 and pets. (Pets is a gene of the mak type.)

Superkiller strains, which produce greater than normal amounts of toxin activity, have been isolated as mutants of a normal killer strain. These mutations are located in at least six different chromosomal genes. These genes may be regulators of killer plasmid copy number.

. . . . . Drs. R. B. Wickner, M. J. Leibowitz, and A. Toh-e

## VI. POLYAMINES

*Mutations in Spermidine Biosynthesis in Escherichia coli and Saccharomyces cerevisiae*

New methods have been developed for the detection of bacterial mutants defective in reactions producing $CO_2$. Although these methods were developed for detection of adenosylmethionine decarboxylase-deficient mutants, they will be of use in finding any mutants where no easy selection system is available.

. . . . . Drs. H. Tabor, C. W. Tabor, and E. W. Hafner

Two general methods have also been developed for markedly increasing the frequency with which Mu-insertion mutations are obtained.

. . . . . Drs. H. Tabor, E. W. Hafner, and C. W. Tabor

With these methods, mutations have been obtained in the various enzymes involved in spermidine biosynthesis (e.g., ornithine decarboxylase, arginine decarboxylase, agmatine ureohydrolase, and adenosylmethionine decarboxylase). Extensive mapping and analytical studies have been carried out on the adenosyldecarboxylase-deficient mutant (called speD). The map position is 2.7 minutes on the E. coli chromosome. No adenosylmethionine decarboxylase or spermidine is present in the Mu-insertion mutants. Despite this complete lack of spermidine, only a small decrease is observed in the growth rate of the mutant.

. . . . . Drs. H. Tabor, C. W. Tabor, and E. W. Hafner

A comparable mutant has also been obtained in S. cerevisiae. No adenosylmethionine decarboxylase or spermidine is present. The growth rate is only slight decreased. Extensive mapping experiments have been carried out.

. . . . . Drs. M. S. Cohn, C. W. Tabor, and H. Tabor

Adenosylmethionine decarboxylase has been purified to homogeneity for yeast and shown to contain one pyruvate per subunit, covalently bound, presumably at the active site. This is the first time this unusual prosthetic group has been found in eukaryotes.

. . . . . Drs. M. S. Cohn, C. W. Tabor, and H. Tabor

Adenosylmethionine decarboxylase has also been purified to homogeneity from liver and shown also to contain one pyruvate per subunit.

. . . . . Drs. A. A. Demetriou, M. S. Cohn, C. W. Tabor, and H. Tabor

Further work has been carried out on our methods, using the amino acid analyzer, for the polyamines and for glutathione and its derivatives. A particularly useful byproduct of these studies has been the development of a specific assay for carboxyglutamate (the amino acid recently described in prothrombin).

. . . . . Drs. C. W. Tabor and H. Tabor

## VII. EVOLUTION

Experiments on the origin of the genetic code have continued and some discriminatory binding between hydrophobic peptides and alkyl uracils has been demonstrated. A nucleic acid-like homopolymer with 5-benzyl uracil as the base has been prepared.

. . . . . Drs. H. M. Chao and S. Black

## VIII. TRAUMATIC SHOCK AND CELLULAR IMMUNITY

Previous studies from this laboratory have shown a defect in cellular immunity of burned mice with a decreased in vitro cytotoxicity of lymphocytes. Investigations into the mechanism of this phenomenon showed no defect of effector lymphocytes. In the present experiments, no defect was found in the sensitization of spleen cells from burned mice when assayed for cytotoxicity, since both responding and stimulating capacities of spleen cells were normal in the one-way mixed lymphocyte culture. In addition, no inhibitor of cytotoxicity in the in vitro sensitization of spleen cells could be found in postburn serum. The mechanism, therefore, remains obscure at the present time.

Other experiments on the role of catecholamines in burn, tourniquet, and endotoxin shock demonstrated that the $\beta$ receptor-blocking drug, propanolol, significantly protected mice against death after burn and tourniquet trauma, while the $\alpha$ receptor-blocking drug, dibenamine, significantly protected mice from endotoxin shock. In burn shock, measurements of the circulation indicated

values closer to normal as early as 5 hours postburn in the propanolol pretreatment group than in the controls. These results suggest that catecholamines which stimulate β receptors produce deleterious effects in traumatic shock.

In other experiments the swelling of ischemic trauma was largely controlled by hydrostatic forces, while in burn trauma osmotic forces played an important role. Application of a vacuum produced a paradoxical early decrease of swelling in burn, but not in tourniquet, trauma.

. . . . . Drs. K. Markley and S. M. Rosenthal and Mrs. E. T. Smallman

## IX. MYCOBACTERIUM

Techniques for quantitative evaluation of the growth of granulocytes were improved by staining the cells for their chloroacetate esterase and peroxidase. The neutrophil granules stained bright red, and eosinophil granules, marked yellow. This method is more accurate than that reported before in which the cells were stained with sudan black.

2-Fluorohistidine showed marked suppressive action on the growth of granulocytes in cultures.

Motion picture studies revealed a very interesting phenomenon of the so-called "novel cells." These cells have been reported to be able to survive for only a few hours in cultures. Under cinemicrography, these cells changed into fast-moving cells and released from the culture surfaces a few hours after cultivation. They exhibited constant rapid protraction and retraction of projections of various forms, ranging from cytoplasmic filaments to wing-like membranes. They moved singly or in small groups like flying insects. The number of cells decreased progressively and disappeared from cultures in a period of 3 to 6 days.

Very long forms, often accompanied with various branches, of Mycobacterium lepraemurium were observed in macrophage cultures maintained at a lower temperature, 30°. At this temperature, the organisms maintained their growth by elongation without multiplication.

. . . . . Dr. Y. T. Chang

ANNUAL REPORT OF THE LABORATORY OF BIOCHEMICAL PHARMACOLOGY

July 1, 1976 through September 30, 1977

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Note: The last two numbers of the project refer to the number of years that the project has been in existence.

Project numbers assigned to this laboratory are ZO1 AM 23,000 LBP to ZO1 AM 24,999 LBP. It is hoped that the above numbering system will allow all projects to be listed alphabetically by Principal Investigator in the future.

Dr. Claude B. Klee's Project No. ZO1 AM 23,930-06 LBP was terminated on 1/19/74.

ANNUAL REPORT OF THE LABORATORY OF BIOCHEMICAL PHARMACOLOGY

July 1, 1976 through September 30, 1977


I. PROTEIN STRUCTURE AND MECHANISM OF ENZYME AND HORMONE ACTION

- Studies on Structure and Function of Thyrotropin Receptor . . . . . Dr. Kohn
- Studies on Relationships between Glycoprotein Hormones and Bacterial Toxins . . . . . Dr. Kohn
- Studies on Mechanism of Action of Interferon . . . . . Dr. Kohn
- Studies on Structure and Function of Receptors for Other Glycoprotein Hormones . . . . . Dr. Kohn
- Tryptophan Synthase of *Escherichia coli* . . . . . Dr. Miles
- Aminoacyl tRNA Synthetases . . . . . Dr. Black

II. SYNTHESIS OF PROTEINS AND NUCLEIC ACID

- Nucleic Acid Synthesis . . . . . Dr. Nossal
- Protein Synthesis and Gene Expression in *Escherichia coli* . . . . . Dr. Furano
- The Mechanism of Action of Nerve Growth Factor (NGF) . Dr. Furano

III. COMPLEX CARBOHYDRATES . . . . . Dr. Ginsburg

IV. THE OUTER MEMBRANE OF *ESCHERICHIA COLI*

- Assembly and Organization of the *Escherichia coli* Outer Membrane . . . . . Dr. Leive
- Permeability Function of the Membranes Studied with Mutants . . . . . Dr. Leive
- Physiology and Genetics of an *Escherichia coli* Strain Important in Studies of Outer Membrane . . . . . Dr. Leive

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- The "Killer Character" of *Saccharomyces cerevisiae* . . Dr. Wickner

VI. POLYAMINES

- Mutations in Spermidine Biosynthesis in *Escherichia coli* and *Saccharomyces cerevisiae* . . . . . Dr. H. Tabor

VII. EVOLUTION . . . . . Dr. Black

VIII. TRAUMATIC SHOCK AND CELLULAR IMMUNITY . . . . . Dr. Markley

IX. MYCOBACTERIUM . . . . . Dr. Chang

SMITHSONIAN SCIENCE INFORMATION EXCHANGE
PROJECT NUMBER (Do NOT use this space)

U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE
PUBLIC HEALTH SERVICE
NOTICE OF
INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER
Z01 AM 23,140-19 LBP

PERIOD COVERED
July 1, 1976 through September 30, 1977

TITLE OF PROJECT (80 characters or less)

Biochemistry of Sulfur-Containing Compounds

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

| | | | |
|---|---|---|---|
| PI: | Simon Black, Ph.D. | Biochemist | LBP NIAMDD |
| | and Chief, Section on Biochemistry of Amino Acids | | LBP NIAMDD |
| OTHER: | Helen M. Chao, Ph.D. | Staff Fellow | LBP NIAMDD |

COOPERATING UNITS (if any)

None

LAB/BRANCH
Laboratory of Biochemical Pharmacology

SECTION
Section on Biochemistry of Amino Acids

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NIAMDD, NIH, Bethesda, Maryland 20014

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| 3.6 | 1.4 | 2.2 |

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

I. Glycyl tRNA synthetase regulation. A study of glycycl tRNA synthetase has revealed that two forms of this enzyme are interconverted in yeast cells as part of the operation of a regulatory mechanism that should be vital in governing the synthesis of proteins. The cyclic interconversion is now being studied *in vitro*.

II. Evolution. Evidence has been obtained that a number of 5'-substituted uracils have a substantial affinity and modest discriminating capacity for certain hydrophobic peptides. A nucleic acid-like homopolymer of one base, 5-benzyl uracil, has been prepared for use in further pursuit of the origin of the genetic code.



FS-040

Project Description:

I. Protein Synthesis. The aminoacyl tRNA synthetases of all living organisms catalyze the following general reaction:

$$\text{amino acid} + \text{ATP} + \text{tRNA} \rightleftharpoons \text{aminoacyl tRNA} + \text{AMP} + \text{PP}.$$

We have found previously that within the yeast cell two forms of the glycyl tRNA synthetase co-exist, one being dependent on activator-like substances, whereas the other is active in the absence of any special additives. These two forms appear to be cyclically interconverted during growth as part of a regulatory mechanism. For several years we have sought to demonstrate this interconversion of the two forms in cell-free extracts as an enzymatic process. The interconversion can be readily observed in extracts but has been difficult to control in a way that permits systematic study. Recent findings indicate that when a change is observed as a presumably single process it is in fact due to at least two independent processes that produce superficially similar test results through different routes. This has caused experiments to be difficult of interpretation and exact replication. With the new picture in view more rapid progress can be reasonably expected.

We plan to continue this study with the general objective of illucidating the detailed components and operation of the mechanism by which the primary step of protein synthesis is regulated.

II. Evolution. Experiments designed to elucidate the origin of the genetic code have been further pursued. One case of particular interest concerns the affinity of a peptide with phenylalanine residues for a uracil with an ethyl-thioethyl side chain in the 5'- position. This uracil binds to very dilute phenylalanine peptide in high ratio but appears to have little affinity for the corresponding peptides of other hydrophobic amino acids. A potential for considerable specificity is therefore evident, especially since the binding patterns of close homologues of the base are very different.

A polyuracil nucleic acid has been prepared in which each base has a benzyl group in the 5'- position. This homopolymer is readily soluble in water despite the large hydrophobic groups, and should be useful in further studies of peptide-template binding.

Publications:

Chao, H. M., Hazel, B., and Black, S.: Binding of uracil derivatives to hydrophobic peptides and sodium dodecyl sulfate. J. Biol. Chem. 251: 6924-6928, 1976.

| SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space) | U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT** | PROJECT NUMBER Z01 AM 23,230-27 LBP |
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PERIOD COVERED
July 1, 1976 through September 30, 1977

TITLE OF PROJECT (80 characters or less)

Chemotherapy of Mouse Leprosy

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: Yao Teh Chang, M.D. Research Pharmacologist LBP NIAMDD

COOPERATING UNITS (if any)

None

LAB/BRANCH
Laboratory of Biochemical Pharmacology

SECTION
Section on Pharmacology

INSTITUTE AND LOCATION
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☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Techniques for quantitative evaluation of the growth of granulocytes were improved by staining the cells for their chloroacetate esterase and peroxidase activity. Neutrophil granules stained bright red and eosinophil granules, marked yellow. Limited growth of megakaryocytes was observed in bone marrow cultures. 2-Fluorohistidine showed marked suppressive action on the growth of granulocytes in cultures.

Motion picture studies revealed a very interesting phenomenon of the so-called "novel cells." These cells changed into fast-moving cells and released from the culture surfaces a few hours after cultivation. They exhibited constant rapid protraction and retraction of projections of various forms, ranging from cytoplasmic filaments to wing-like membranes. The number of cells declined progressively, and the cells disappeared from cultures in a period of 3 to 6 days.

Very long forms, often accompanied with various branches, of Mycobacterium lepraemurium were observed in macrophage cultures maintained at a lower temperature (30°). Studies of a combination of two antiamebic drugs, broxyquinoline and broxaldine in murine leprosy revealed little suppressive activity.

Project Description:

Quantitative Estimation of Granulocytes. Techniques for quantitative evaluation of the growth of granulocytes were improved by staining the cells for their chloroacetate esterase and peroxidase activity. Neutrophil granules stained bright red and eosinophil granules, marked yellow. This method is more accurate and time saving than that reported before in which the cells were stained with sudan black.

Granulocytes showed better growth at a lower temperature of 33° than at 37°. At 37°, the cells showed numerous vacuoles in the cytoplasm of the more advanced forms of granulocytes, while at 33° there were practically no vacuoles in the cytoplasm. The lower temperature may be used for future quantitative studies of granulopoiesis.

Growth of Megakaryocytes and Eosinophils. Limited growth of megakaryocytes was observed in bone marrow cultures maintained in a medium containing horse serum and medium 135, omitting the regular supplement of bovine embryo extract as used in the past. Mitosis of megakaryocytes and liberation of platelets were frequently observed in the culture over a period of one week. Eosinophils were also observed in the cultures, in about 10% of the total number of granulocytes. The number declined progressively, and the cells disappeared in about one week.

Purification of the Granulocyte Colony-Stimulating Factor (CSF). Partial purification by DEAE-cellulose chromatography of the CSF was made from the endotoxin-induced mouse serum. Preliminary studies showed very encouraging results. The experiment was interrupted because of lack of personnel.

Cinemicrography of the "Novel Cell". Motion picture studies revealed a very interesting phenomenon of the so-called "novel cells." These cells have been reported to be able to survive for only a few hours in cultures. Under our cinemicrography, these cells changed into fast-moving cells and released from the culture surfaces a few hours after cultivation. They exhibited constant rapid protraction and retraction of cytoplasmic projections of various forms ranging from filaments to wing-shaped membranes. They moved singly or in small groups. The forms of cells changed rapidly simulating figures of various animals or insects, sometimes like flying mosquitoes. Although the function of these cells is unknown, they could be of an important new type of cells, as judged from their larger numbers present in the spleen cell suspensions.

Growth of Mycobacterium lepraemurium in Macrophages at Low Temperature. Very long forms, often accompanied with various branches, of M. lepraemurium were observed in macrophage cultures maintained at a lower temperature (30°). At this temperature, the organisms maintained their growth by elongation without multiplication.

Chemotherapy of Murine Leprosy. The effect of two antiamebic drugs, broxyquinoline and broxaldine, was studied in murine leprosy in mice. The negative results of the drugs tested singly were reported last year. Studies of the effect of a combination of the two drugs also showed no effect. These drugs have been reported to show some effect in the treatment of leprosy in India.

Publications:

Nossal, R. J., and Chang, Y. T.: A procedure for determining mobility parameters for cells moving along surfaces. J. Mechanochem. Cell Motility 3: 247-251, 1976.

Yamagami, A., and Chang, Y. T.: Growth of Mycobacterium lepraemurium in cultures of macrophages obtained from various sources. Infect. Immun., in press.

| SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space) | U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT** | PROJECT NUMBER Z01 AM 23,580-14 LBP |
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PERIOD COVERED
July 1, 1976 through September 30, 1977

TITLE OF PROJECT (80 characters or less)

Protein Synthesis and Gene Expression in Escherichia coli and Other Organisms

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: Anthony V. Furano, M.D. Medical Officer (Research) LBP NIAMDD

COOPERATING UNITS (if any)

Dr. Lloyd Greene, Department of Neuropathology, Children's Hospital Medical Center, Boston, Massachusetts

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NIAMDD, NIH, Bethesda, Maryland 20014

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

We are continuing our investigations into the regulation of protein synthesis and gene expression in Escherichia coli. In addition, we have extended our studies to examine the mechanism whereby nerve growth factor (NGF) induces the differentiation of immature neural cells into mature neurons. The studies on NGF are being carried out with the collaboration of Dr. Lloyd Greene.

Project Description:

I. Protein Synthesis and Gene Expression in Escherichia coli. We recently showed that although the concentration of the peptide chain elongation factor EF-Tu is maintained at an 8- to 14-fold molar excess above the concentration of ribosomes or EF-G or EF-Ts, the synthesis of EF-Tu is nonetheless coordinated with that of ribosomes EF-G and EF-Ts. We are now investigating two questions that arise from these findings: How does the cell synthesize so much EF-Tu? How does the cell coordinate the synthesis of EF-Tu with that of ribosomes and other macromolecules?

Pertaining to the first question, Nomura and coworkers recently showed that the cell contains at least two genes for EF-Tu (tufA and tufB), and work from this laboratory has recently shown that the EF-Tu's coded by these two genes are identical except for one peptide. Thus, unlike the only other example of a duplicated gene for a protein in Escherichia coli, ornithine carbamyl transferase, the tufA and tufB genes have hardly diverged. This result suggests that the duplication of the tuf gene may serve only to maintain the high synthetic rate of EF-Tu. As was pointed out earlier, a high level of EF-Tu is consistent with its role in protein synthesis, which is to form a ternary complex with GTP and aminoacyl tRNA. It is in this form that aminoacyl tRNA gains access to the ribosome, and the amount of EF-Tu is approximately equimolar with that of aminoacyl tRNA at all growth rates. This fact prompted us to examine the relationship of EF-Tu and aminoacyl tRNA in crude extracts, and studies led us to the discovery of a new factor(s) in crude extracts which dissociates the aminoacyl tRNA from the ternary complex.

Our investigations of how the cell coordinates the synthesis of EF-Tu with the other components of the protein synthetic apparatus led us to the finding that like tRNA, rRNA, and ribosomal proteins, EF-Tu and EF-G are under stringent control, i.e., expression of the relA$^+$ allele causes a decrease in the synthesis of EF-Tu and EF-G relative to that of other proteins. We also showed the rather surprising result that the synthesis of a large number of proteins are influenced by the relA gene. We have continued these studies to show that during conditions which produce expression of the relA allele, derepression of the tryptophan biosynthetic pathway, but not the arginine biosynthetic pathway, is decreased in a relA$^-$ strain. In contrast, derepression of the arginine biosynthetic pathway, but not the tryptophan biosynthetic pathway, is decreased in an otherwise isogenic relA$^+$ strain. The latter finding suggests that the arginine biosynthetic enzyme is under stringent control, perhaps because this pathway provides the biosynthetic intermediates for the synthesis of polyamines which interact with many components of the protein synthetic apparatus.

II. The Mechanism of Action of Nerve Growth Factor (NGF). In collaboration with Dr. Lloyd Greene, we are studying the effect of the protein NGF on a line of cells cloned from a murine neural tumor. In the absence of NGF, the cells grow as small, round cells devoid of any of the histological and many of the biochemical features of neural cells. Upon the addition of NGF, essentially all of the cells stop dividing and after a period of differentiation assume most of the histological, physiological, and biochemical properties of a mature neural cell. We are currently analyzing to what extent the population

of proteins synthesized by the differentiated cells differs from that synthesized by cells growing without NGF, at what time during differentiation the changes in protein synthesis occur, and by what means NGF produces these changes.

Publications:

Furano, A. V.: Dissociation of aminoacyl tRNA from the complex of EF-Tu--GTP--aminoacyl tRNA by extracts of Escherichia coli. Biochem. Biophys. Res. Commun. 76: 309-316, 1977.

Furano, A. V.: The elongation factor Tu coded by the tufA gene of Escherichia coli K-12 is almost identical to that coded by the tufB gene. J. Biol. Chem. 253: 2154-2157, 1977.

Furano, A. V., and Wittel, F. P.: Effect of the relA gene on the synthesis of individual proteins in vivo. Cell 8: 115-122, 1976.

Furano, A. V., and Wittel, F. P.: The effect of the relA gene on derepression of amino acid biosynthetic enzymes in growing Escherichia coli depends on the pathway being derepressed. J. Bacteriol., in press.

| SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space) | U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT** | PROJECT NUMBER Z01 AM 23,630-12 LBP |
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PERIOD COVERED
July 1, 1976 through September 30, 1977

TITLE OF PROJECT (80 characters or less)

Biology of Complex Carbohydrates

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

| | | | |
|---|---|---|---|
| PI: | Victor Ginsburg, Ph.D. | Research Chemist | LBP NIAMDD |
| | and Chief, Section on Biochemistry | | LBP NIAMDD |
| OTHERS: | David A. Zopf, M.D. | Senior Staff Fellow | LBP NIAMDD |
| | Chao-Ming Tsai, Ph.D. | Senior Staff Fellow | LBP NIAMDD |
| | David F. Smith, Ph.D. | Senior Staff Fellow | LBP NIAMDD |

COOPERATING UNITS (if any)

None

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| 4.0 | 3.6 | 0.4 |

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Oligosaccharides will be isolated from natural sources. Oligosaccharides will be coupled to macromolecules in order to make antibodies specific for certain sugar sequences. The antibodies will be used in radioimmune assays for the occurrence of these sugar sequences in the glycolipids and glycoproteins of cell membranes and serum in order to investigate the involvement of the complex carbohydrates of cell surfaces in development and regulation or as tumor-specific antigens.

Waldenström macroglobulins and myeloma proteins will be screened for their ability to react with human erythrocytes. Those that do will be tested for their ability to react with various oligosaccharides coupled to macromolecules in order to define their specificity.



PHS-6040

Project Description:

Many carbohydrates of cell membranes occur at levels too low to be detected by conventional means. We are developing immunological methods for their detection using two general approaches for obtaining carbohydrate-specific antibodies: (1) animals will be immunized with pure oligosaccharides coupled to macromolecules and (2) the specificities of Waldenström macroglobulins and myeloma proteins which bind carbohydrates will be determined. Examples of both of these approaches are given in the following paragraphs.

A purified human monoclonal IgM(κ) (agglutinin MKV) has been characterized as both a cold agglutinin and a cryoglobulin. As its reactivity with human erythrocytes is reduced by treatment of the cells with ficin and its reactivity with both is abolished by treatment of the cells with neuraminidase, it has by definition blood group $Pr_2$ specificity. The membrane receptors for agglutinin MKV are sialic acid-containing glycoproteins in human cells and sialic acid-containing glycolipids in dog cells. Agglutination of erythrocytes is specifically inhibited by gangliosides $GM_3$ and N-acetylneuraminyl-paragloboside but not by their N-glycolylneuraminyl forms or by lactosyl ceramide or paragloboside, and the reactivity of neuraminidase-treated cells can be restored by allowing them to absorb either $GM_3$ or N-acetylneuraminyl-paragloboside. When large amounts of gangliosides are absorbed, the cells are agglutinated not only in the cold but also at 37°, showing that the density of receptor sites on the erythrocyte surface can influence the thermal amplitude of cold agglutinins and supporting our previous suggestion that cold agglutinins in general are simply low affinity antibodies which require multivalent attachment for the stabilization of bonds strong enough to agglutinate erythrocytes. Liberation of sialic acid from cold agglutinin MKV by treatment with neuraminidase does not affect its agglutinating properties, but the asialoprotein is no longer a cryoglobulin. Apparently the physical basis for its precipitation in the cold is the intermolecular immune binding of N-acetylneuraminyl residues.

Aminophenylethylamine derivatives of oligosaccharides conjugated to the hemp seed protein, edestin, are effective antigens. Rabbits immunized with the conjugates produce antibodies that bind radiolabeled oligosaccharides. Specificity of binding is determined by comparing inhibitory activities of structurally-related oligosaccharides in radioimmunoassays. All of three rabbits immunized with a mannotetraose derivative, two of five rabbits immunized with a lacto-N-fucopentaose III derivative, and four of five rabbits immunized with a lacto-N-tetraose derivative, developed useful antibodies (i.e., antibodies with dissociation constants for the corresponding oligosaccharide of less than $10^{-5}$). These antibodies can be used to detect specific sugar sequences in complex carbohydrates.

Proposed Course:

Oligosaccharides will be isolated from natural sources. Certain oligosaccharides will be coupled to macromolecules in order to make antibodies specific for certain sugar sequences. The antibodies will be used in radioimmune assays for the occurrence of these sugar sequences in the glycolipids and glycoproteins of cell membranes and serum.

Waldenström macroglobulins and myeloma proteins will be screened for their ability to react with human erythrocytes. Those that do will be tested for their ability to react with various oligosaccharides coupled to macromolecules in order to define their specificity.

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| SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space) | U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT** | PROJECT NUMBER Z01 AM 23,960-11 LBP |
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PERIOD COVERED
July 1, 1976 through September 30, 1977

TITLE OF PROJECT (80 characters or less)

Cell Regulation by the Action of Pharmacodynamic Agents on the Cell Membrane

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

| | | | |
|---|---|---|---|
| PI: | Leonard D. Kohn, M.D. | Senior Surgeon, USPHS | LBP NIAMDD |
| | and Chief, Section on Biochemistry of Cell Regulation | | LBP NIAMDD |
| OTHERS: | Brian R. Mullin, M.D. | Senior Staff Fellow | LBP NIAMDD |
| | Salvatore M. Aloj, M.D. | Visiting Scientist | LBP NIAMDD |
| | George Lee, Ph.D. | Staff Fellow | LBP NIAMDD |
| | Evelyn F. Grollman, M.D. | Medical Officer (Research) | LBP NIAMDD |

COOPERATING UNITS (if any)

Please see next page.

LAB/BRANCH
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SECTION
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| TOTAL MANYEARS: | PROFESSIONAL: | OTHER: |
|---|---|---|
| 5.8 | 4.7 | 1.1 |

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Studies indicate that there is a structural and functional relationship in the mechanism by which glycoprotein hormones [thyrotropin (TSH), luteinizing hormone (LH), human chorionic gonadotropin (hCG), and follicle-stimulating hormone (FSH)], certain bacterial toxins (cholera and tetanus, for example), and the antiviral protective agent, interferon, transmit their message through the cell membrane. Thus, in each case these agents appear to be composed of two subunits or regions in their molecular structure, i.e., the α and β subunits of the glycoprotein hormones or the A and B proteins of cholera toxin. One subunit or region, the β subunit or B protein of the hormones and cholera toxin, for example, interacts with a receptor containing a ganglioside or ganglioside-like molecule whose oligosaccharide determinant causes a specific conformational change in the hormone molecule; interaction with a receptor containing the wrong oligosaccharide moiety will not cause this conformational change and will therefore not allow further message transmission. As a consequence of the initial interaction

(continued on next page)

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SUMMARY OF WORK (continued)

of the first subunit, as a consequence of the second subunit translocation, or as a consequence of both events together, a change in membrane state results. The change in membrane state expresses itself as a different exposure of cell surface determinants, including those important as receptors for other effectors of cell function and as a change in the electrochemical gradient across the membrane. Finally, as a consequence of some or all of these preceding events, changes in cyclic AMP levels, which are effected by modulating the activity of the adenylate cyclase system, transmit the effector message to the translational and transcriptional machinery of the cell.

Project Description:

Objectives:

The thrust of work in this section is toward an understanding of the mechanisms regulating cell activity. Special emphasis is on studies concerning the action of pharmacodynamic agents on enzymes within the cell and in its membrane. Specifically, studies have been initiated which concern the mechanism of exophthalmos and Graves' disease, preventive therapeutic measures for these diseases, and hormone receptor function at a membrane level. These studies have been extended to the enzymatic mechanisms of membrane transport in bacterial and mammalian cells; to membrane structure; to enzymatic mechanisms in the biosynthesis of collagen and their relation to human collagen disease states; and to the structure and function of oxido reductases important to energetic regulation of cell functions.

Major Findings:

I. The Role of Gangliosides in the Thyrotropin (TSH) Receptor. Gangliosides are a family of glycolipids whose nomenclature is derived from the length of the oligosaccharide core structure ($G_{M1}$, $G_{M2}$, $G_{M3}$) as well as the number and location of sialic acid residues within the oligosaccharide moiety, i.e., mono-, di-, and trisialic acid derivatives ($G_M$, $G_D$, and $G_T$, respectively) are further defined isomerically ($G_{D1a}$, $G_{D1b}$, $G_{T1}$) on the basis of the galactose residue to which the sialic acid residues are linked. The ganglioside $G_{M1}$ is not only a component of the specific receptor for cholera toxin, its interaction with toxin is presumed to initiate a sequence of events resulting (i) in the dissociation of the A from the B protein of the toxin and (ii) in the formation of an activated $A_1$ subunit which translocates within the cell membrane and activates the adenylate cyclase system.

Our studies have shown that the gangliosides $G_{D1b}$ and $G_{T1}$ are potent inhibitors of TSH binding to its specific membrane receptor; that this inhibition is caused by an interaction of these gangliosides with the hormone rather than the membrane; that the inhibition is hormone-specific; and, most important, that these gangliosides appear to impose on the TSH molecule a conformation distinct from that resulting from the interaction of TSH with gangliosides which are poor inhibitors of TSH binding, i.e., $G_{D1a}$, the structural isomer of $G_{D1b}$. These results led to the suggestion that TSH had a receptor structure similar to that of cholera toxin and that both effectors were analogous in their message transmission process. Specifically, the following was suggested: the $\beta$ subunit of TSH interacts with receptor containing a ganglioside as a functional component; the ganglioside confers hormone or toxin specificity through its oligosaccharide determinant; the "correct" oligosaccharide determinant causes a unique conformation in its particular effector which is necessary for the $\alpha$ subunit to translocate within the membrane; the $\alpha$ subunit of TSH, like the A protein of cholera toxin, carries information on its primary sequence necessary for the stimulation of the adenylate cyclase system; and, last, cholera toxin and TSH might not only have a common membrane component with which they interact, but differences in such interactions might explain the toxicity of one and the regulatory role of the other.

Among the experimental evidence which has accumulated to support these predictions are the following: Thyroid plasma membranes are not only rich in gangliosides with the chromatographic characteristics of $G_{D1b}$, $G_{T1}$, and $G_{M1}$, but also in higher order ganglioside which (i) are even more potent inhibitors of TSH binding and (ii) do not appear to be present in the brain. A TSH receptor defect in a rat thyroid tumor has been correlated with an alteration in the ganglioside content of the membranes of this tumor and with a defect in the biosynthetic enzymes concerned with the synthesis of the higher order gangliosides implicated as components of the TSH receptor structure. The ganglioside $G_{M1}$ induces a conformational change in cholera toxin which cannot be duplicated by other gangliosides and cholera toxin interacts with thyroid plasma membranes, these interactions affecting the ability of TSH both to bind to the membranes and to stimulate the adenylate cyclase activity of the membranes.

Regarding the adenylate cyclase stimulation, it is to be noted that NAD significantly enhances the ability of cholera toxin to stimulate adenylate cyclase activity in thyroid plasma membranes, whereas it inhibits the ability of TSH to stimulate adenylate cyclase activity in these same thyroid membranes. This finding suggests that cholera toxin and TSH diverge in the molecular mechanism of adenylate cyclase stimulation and that there exist intermediate steps (involving NAD) between receptor recognition and adenylate cyclase stimulation.

Last, but far from least, support for the schema comes from the fact that sequence homologies have been demonstrated to exist between cholera toxin, TSH, and the other glycoprotein hormones. Regarding the homology between the B protein of cholera toxin and the β subunits of the TSH-LH-hCG-FSH superfamily, it is especially notable (i) that the CAGY region of the glycoprotein hormones represents an important homology which has been highly preserved despite structural and functional differences which occurred in their evolution of progression to specialized target organ functions; (ii) that this homology exists on that portion of the glycoprotein or toxin molecules, the β subunit or the B protein, which carries the primary determinants for receptor binding; and (iii) that the β subunits of the glycoprotein hormones determine their target organ specificity. This region of sequence similarity is thus an obvious candidate for the "active site" concerned with binding to receptors on the cell membrane.

Support for this interpretation is offered by two recent observations. First, a synthetic decapeptide, including the CAGY sequence and the three residues on either side in the TSH sequence, will inhibit TSH binding and stimulate cholera toxin binding, i.e., have an effect similar to TSH itself. Second, trypsin, a protease with a CAGY sequence, can inhibit TSH binding to the intact and solubilized TSH receptor without destroying TSH receptor activity.

In regard to the sequence homology noted between the A protein of cholera toxin and α subunit of the glycoprotein hormones, this region is startlingly similar in sequence to the nonapeptide neurohypophyseal hormones, oxytocin and vasopressin, i.e., to nonapeptides whose action also involves adenylate cyclase stimulation.

II. A Change in the State of the Membrane Results from the Interaction of TSH or Cholera Toxin with Its Specific Receptors. A most important experiment concerning the cholera toxin-TSH interaction with thyroid plasma membranes came from the attempt to show by direct biochemical means that $G_{M1}$ is the cholera toxin receptor on thyroid plasma membranes. Thyroid membranes incubated with galactose oxidase, followed by exposure to sodium borotritide, should have tritium incorporated into ganglioside residues if these residues are available to the enzyme. When such an experiment was performed, the major labeled ganglioside of thyroid membranes was $G_{M1}$. As predicted by the hypothesis that $G_{M1}$ is involved in the cholera toxin receptor structure, the same experiment performed on membranes pretreated with cholera toxin showed that the $G_{M1}$ residues were not labeled with tritium. Most important, however, was the observation that pretreatment with cholera toxin caused an enhancement of the labeling of other glycolipids contained in the membrane which chromatographed in the region of $G_{D1b}$, $G_{T1}$, $G_{M2}$, and $G_{M3}$, i.e., caused an exposure of surface determinants important as receptors for other effectors of cell function.

This last observation was of special interest in the light of the data which showed that low concentrations of cholera toxin enhanced TSH binding to thyroid plasma membranes and that there was a cooperative relationship between ganglioside or ganglioside-like receptors for the different effectors, TSH and cholera toxin. Since analogous data have been obtained when TSH interacts with the cell membrane, it appears that a change in the state of the membrane is a general feature of the events immediately following the receptor-effector interaction.

III. The Role of Ganglioside or Ganglioside-Like Compounds in the Structure and Function of Receptors for Other Glycoprotein Hormones. The sequence homologies between LH, hCG, FSH, TSH, and cholera toxin raised the obvious possibility that all the glycoprotein hormones would have a similar receptor structure and a similar mechanism of receptor interaction. Each target organ would thus have as its receptor a ganglioside or ganglioside-like structure with a specific and unique carbohydrate sequence, i.e., one which was different from that on other target organs. The interaction of the appropriate hormone with this specific oligosaccharide would result in a unique conformational shift such that the $\alpha$ subunit would be placed in the position favored for membrane translocation and adenylate cyclase activation in that particular target tissue. Interaction with the wrong hormone would result in a different conformation, an unfavorable position for membrane translocation, and no transmission of its "message" to the cell membrane. A study of ganglioside inhibition of hCG binding to testes membranes provides preliminary support for a portion of this hypothesis in that the data show that the oligosaccharide moiety of the hCG receptor structure is distinct from the oligosaccharide moiety on the TSH receptor, i.e., the most effective inhibitors of hCG binding, have a terminal sialic acid on the oligosaccharide chain, whereas the most effective inhibitors of TSH binding have a disialyl group linked to the internal galactose residue. Studies with LH are consistent with these conclusions in that gangliosides also inhibit LH binding to testes membranes, the ganglioside specificity is distinct from that of both hCG and TSH, and the different gangliosides impose distinct conformational changes on hCG and LH which can be correlated with the inhibition data.

IV. Similarities in the Structure and Function of Receptors for Cholera Toxin and the Glycoprotein Hormones Extend to Interferon. Evidence has accumulated which suggests that the binding of interferon to specific cell surface receptors is necessary for the development of its antiviral action and that these receptors, like the glycoprotein receptors above, contain gangliosides or ganglioside-like structures whose oligosaccharide moiety is a critical feature of the receptor structure. In this regard, experiments have shown that cholera toxin and TSH inhibit the establishment of the antiviral state by interferon; these experiments were based on the hypothesis that cholera toxin or TSH might directly or indirectly interfere with the gangliosides or ganglioside-like oligosaccharide structures believed to be important for interferon's interaction with its cell membrane receptors. Direct in vitro binding experiments with cholera toxin and TSH indicate that this notion is valid and establish that the antiviral action of interferon is a membrane-initiated event.

Experiments using TSH or cholera toxin as probes of the mechanism of interferon action also indicate the following: (i) The initial binding of interferon to a ganglioside or ganglioside-like receptor is insufficient to establish the antiviral state, i.e., human KB-3 cells which are unresponsive to either mouse or human interferon can bind both. (ii) The receptor for interferon has both a glycoprotein and a ganglioside as its structural components. (iii) Immediately following the interaction of interferon with this receptor structure, there is a change in membrane state analogous to that seen when cholera toxin and TSH interact with thyroid membranes; cells nonresponsive to interferon but able to bind interferon (human KB-3 cells) do not have the same change. (iv) The failure of human KB-3 cells to transmit the interferon message to the cell machinery, despite the ability of interferon to bind to the cell surface of the cells, correlates with a near absence of membrane gangliosides, especially $G_{M2}$ and $G_{M3}$, which are present in high levels in membranes of sensitive mouse L cells. (v) Interferon stimulates adenylate cyclase activity in sensitive cells, maximal stimulation preceding the establishment of the antiviral state. (vi) Interferon induces changes in the electrochemical gradient across the cell membrane; this effect may be related to the ability of ouabain to inhibit interferon action (see below).

V. Similarities in the Structure and Function of Receptors for Cholera Toxin and the Glycoprotein Hormones Extend to Other Bacterial Toxins and This Relationship Has Pathophysiological Significance. The above observations raised the possibility that bacterial products other than cholera toxin with a known tropism for gangliosides such as tetanus toxin and Escherichia coli enterotoxin might interact with thyroid plasma membranes, and even with the TSH receptor on the membranes. In this regard, tetanus toxin is of particular interest, since the specificity of the ganglioside-tetanus interaction ($G_{D1b} = G_{T1} > G_{M1} > G_{D1a} > G_{M2}$) is similar to the specificity of the ganglioside-TSH interaction and since the clinical presentation of tetanus includes a syndrome which closely resembles thyroid storm.

Tetanus toxin has been shown to interact with thyroid plasma membranes. More important, the properties of this interaction closely resemble the properties

of the TSH interaction with TSH receptors on these membranes; the interaction of tetanus toxin with thyroid membranes exhibit several characteristics of its interaction with neural tissue; tetanus toxin does not bind to membranes of a thyroid tumor with a TSH receptor defect; and tetanus toxin stimulates thyroid hyperfunction in mice subjected to conditions known to precipitate thyroid storm.

VI. The Mechanism by Which Glycoprotein Hormones, Interferon, and the Bacterial Toxins Effect Cell Changes. Current views regarding the mechanism of action of TSH and other glycoprotein hormones invoke alterations in the concentration of cyclic AMP as the second event in the transfer of information from the hormones to the appropriate cell. That is, the information carried by the hormone is purportedly translated into a "second message" by means of a change in intracellular cyclic AMP concentration.

One implication of the schema outlined above is that the increase in intracellular cyclic AMP may not necessarily represent a second message *per se*, but rather an event which is sequentially or simultaneously coupled to a primary effect of these agents on the membrane. In other words, the common denominator of all of the effectors may be an alteration in the membrane which leads to generalized effects on cellular metabolism, one of which may be an increase in the concentration of intracellular cyclic AMP. Viewed in this manner, it is clear that one means of accomplishing this end could be through alterations in electrochemical ion gradients across the cell membrane. In this regard, the following observations are notable: (i) The primary effect of tetanus toxin is an alteration in neural transmission; (ii) cholera toxin causes a dramatic loss of electrolytes and water through the intestinal epithelium; (iii) TSH increases the rate and extent of iodide uptake by thyroid cells; (iv) oubain, the classic inhibitor of sodium, potassium-stimulated ATPase, blocks the antiviral action of interferon; and (v) electrochemical ion gradients play a primary role in active transport and metabolism in prokaryotes, eukaryotes, and many subcellular organelles.

A recent development is the demonstration that TSH causes an increase in the uptake of the lipophilic cation triphenylmethylphosphonium ($TPMP^+$) when added to cultured thyroid cells or to membrane vesicles derived from these cells under appropriate conditions. This effect is dependent upon a specific interaction of TSH with its receptor at the cell surface. Since uptake of $TPMP^+$ and other lipophilic cations has been shown to reflect the presence of an electrical potential across the membrane (interior negative), it is tempting to speculate that one of the primary effects of TSH is to hyperpolarize the thyroid cell membrane.

The effect of TSH on $TPMP^+$ uptake precedes the effect of the hormone on adenylate cyclase activity. The possibility thus exists that a primary mode of action of each of these effectors is to alter electrochemical ion gradients across the cell membrane.

VII. Structural Features of the Receptor. Studies have shown that the two components of thyroid plasma membranes known to interact with TSH, the glycoprotein with specific TSH binding activity, and the gangliosides of

the thyroid membranes, segregate differently when membranes are solubilized with lithium diiodosalicylate. The possibility raised in this study was that the $^{125}$I-TSH binding activity of the intact plasma membrane is contributed to by both the glycoprotein and the ganglioside components of the membrane. Thus, the salt sensitivity of the intact plasma membrane receptor as well as its high degree of hormonal specificity in regard to other glycoprotein hormones (LH and hCG, for example) appears to reflect the ganglioside-TSH interaction. The conclusion concerning the salt sensitivity of the TSH-ganglioside interaction has been confirmed in direct $^{125}$I-TSH binding experiments using ganglioside-containing liposomes. The high degree of hormonal specificity is evident in the close correlation of inhibition of binding with the conformational changes effected in the different hormones by the different gangliosides (see above) and is in contrast to the loss of specificity for LH and hCG exhibited by the glycoprotein receptor fragment.

In sum, these data coupled with previous observations concerning (i) the protease sensitivity of TSH binding and cyclase stimulation in cultured thyroid cells and (ii) the loss of TSH binding and cyclase stimulation in a thyroid tumor whose membranes are deficient in their ganglioside content, suggest that each component contributes to the function of the intact plasma membrane receptor and that the functional transmission of the TSH message to the thyroid cell machinery requires the presence of both a glycoprotein and glycolipid component in the TSH receptor structure. The existence of membrane glycoproteins able to specifically bind the effector is not unique to TSH; thus, they have been shown for hCG and interferon (in human KB-3 cells).

VIII. Studies Concerning Centrifugation. As a cooperating unit, centrifugation studies were performed for this and other Institutes (Paul Torrence, Allen N. Schechter, Edith W. Miles, Thressa C. Stadtman) and for Othmar Gabriel (Georgetown University).

Significance to Biomedical Research and the Progress of the Institute:

The details of the schema outlined in the introduction of this review are not defined for each individual agent; however, the relationships are increasingly clear. The implications to the pathophysiology of Graves' disease and the mechanism of action of cholera and tetanus toxin are already obvious, and it is clear they will extend to other problems. Thus, the observation that contraceptive programs involving the development of antibodies to hCG have also caused significant levels of antibodies to tetanus in some patients, may be the tip of an iceberg, i.e., the implications will extend even to a "normal" disease such as pregnancy. In regard to medicinal chemistry, these relationships clearly imply caution. Yet it is hoped they will hold new opportunities to the development of peptide and carbohydrate analogs which will either block the action of cell membrane effectors which can cause a disease (cholera or tetanus toxin) or mimic the action of effectors regulating body functions (glycoprotein hormones) and blocking viral infestation of the cell (interferon).

The ability of tetanus toxin to interact with thyroid membranes and with TSH receptors on these membranes, as suggested above, has several significant implications and applications. An obvious clinical implication is that the syndrome of sympathetic overactivity which can appear in tetanus is contributed to by the ability of the toxin to induce thyroid hyperfunction. This syndrome would thus be analogous in its etiological relationship to the thyroid as is thyroid storm or as is the hypersensitivity to catecholamines demonstrable in thyrotoxic patients. A second implication is that tetanus toxin will have a receptor structure and mechanism of effecting cell processes analogous to cholera toxin, the glycoprotein hormones, and interferon. Last, it seems reasonable to presume that tetanus toxin interactions with the TSH receptor on thyroid plasma membranes are analogous to its interactions with neural tissue membranes and that studies which compare the tetanus toxin effects on thyroid membranes with those of TSH will clarify both the mechanism by which tetanus exerts its neurotoxic effects and TSH its thyroid-stimulating effects.

Proposed Course:

Studies of the TSH-EPF relationship will be continued in an attempt to examine the mechanism by which EPF activity is generated in humans in vivo. The proposed diagnostic mechanism for human exophthalmos will be further tested as will the therapeutic program. The structure of glycoprotein hormone receptors and the mechanism of action of glycoprotein hormones will be further clarified. The mechanism of procollagen peptidase formation and control will be related to normal and disease states. The biosynthesis of type I procollagen and type III procollagen will be studied. The enzymatic mechanism of active transport in bacteria will be further defined, and studies on thyroid cell transport will be continued. The mechanism of interferon interaction with cell membranes will be further defined as will the relationship of the glycoprotein hormones to the following bacterial toxins: cholera toxin, botulinus toxin, tetanus toxin, and various enterotoxins produced by gram-negative bacteria. The relationships and implications to Graves' disease, exophthalmos, thyroid storm, tetanus, cholera, botulism, etc., will be pursued.

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Winand, R. J., Wadeleux, P. A., Etienne-Decerf, J., and Kohn, L. D.: Biochemical basis of thyroid stimulation in using thyroid cells in tissue culture. In von zur Mühlen, A., and Schleusener, H. (eds.): Biochemical Basis of Thyroid Stimulation and Thyroid Hormone Action. Stuttgart, Germany, Georg Thieme Publishers, pp. 1-23, 1976.

Honors and Awards:

Dr. Leonard D. Kohn was awarded the United States Public Health Service Meritorious Service Medal on May 13, 1977.

| SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space) | U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT** | PROJECT NUMBER Z01 AM 24,020-14 LBP |
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PERIOD COVERED
July 1, 1976 through September 30, 1977

TITLE OF PROJECT (80 characters or less)

Studies on the Chemical and Physiological Properties of the Surface of E. coli

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

| | | | |
|---|---|---|---|
| PI: | Loretta Leive, Ph.D. | Research Biologist | LBP, NIAMDD |
| OTHERS: | William G. Coleman, Jr., Ph.D. | Senior Staff Fellow | LBP, NIAMDD |
| | David Filer, Ph.D. | Visiting Fellow | LBP, NIAMDD |

COOPERATING UNITS (if any)

Dr. Shlomo Rottem, The Hebrew University, Jerusalem, Israel
Dr. Manfred E. Bayer, Institute for Cancer Research, Philadelphia, Pennsylvania

LAB/BRANCH
Laboratory of Biochemical Pharmacology

SECTION
Section on Pharmacology

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20014

| TOTAL MANYEARS: | PROFESSIONAL: | OTHER: |
|---|---|---|
| 3.4 | 2.7 | 0.7 |

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The structure and assembly of the outer membrane of Escherichia coli is being studied. Lipopolysaccharide is inserted at points in the membrane and, after a period when it can diffuse, is organized into domains that cannot be penetrated with new molecules, as shown by density labeling, immunologic labeling, and electron microscopy. Electron spin resonance studies show that the polysaccharide confers rigidity upon the bilayer of this membrane.

Mutants showing antibiotic hypersensitivity due to presumed outer membrane changes have been isolated, mapped, and characterized physiologically. Biochemical characterization is underway.

Conjugation and transduction procedures have been worked out for a strain of E. coli of medical and biochemical interest which has heretofore been refractory to such analysis. The properties of a transducing phage carried by this strain have also been studied.



PHS-6040

Project Description:

I. Structure, Assembly, and Function of the Escherichia coli Outer Membrane. The outer membrane is the outermost layer of the surface of E. coli. It is relatively simple in composition, is assembled from molecules of protein, lipid, and lipopolysaccharide (LPS) made elsewhere in the cell, and therefore serves as a good model for studying membrane structure and assembly.

There are about 10-50 insertion points in the outer membrane into which newly synthesized LPS is inserted, and from which it moves elsewhere in the membrane, as shown by work in this laboratory and others. In current experiments we have shown by density labeling that after this initial period the LPS becomes immobilized into domains that do not mix with subsequently incorporated LPS. By immunological methods it has been possible to show that these domains comprise very approximately 0.1% to 1% of the cell surface in area, each containing approximately 1,000-10,000 LPS molecules. The nature of these domains are being further analyzed.

Freeze-etch electron microscopy of cells treated with EDTA to remove half the LPS shows areas that are unchanged, in thickness and fracture properties, and other areas that are changed. These areas form patches of about 1% of the cell surface. Such results also suggest heterogeneity of composition of different areas within the outer membrane.

The outer membrane is known to be a molecular sieve which admits some molecules and excludes others. To study this barrier function further we have isolated novobiocin supersensitive mutants. Two classes have been characterized: One class maps at minute 79 on the E. coli chromosome, shows increased permeability to a wide range of hydrophobic antibiotics and apparently has some changes in composition of its LPS. Another, which maps at minute 11 on the E. coli chromosome, shows increased permeability to both hydrophobic and hydrophilic antibiotics and other substances, and has no obvious LPS changes. The protein and lipid composition of the outer membrane of these mutants, as well as a detailed study of the LPS, is continuing.

Several reports have suggested that lipid mobility in the outer membrane may be much more restricted than in the cytoplasmic membrane of these and other cells. To determine whether the LPS of this membrane affects the mobility, electron spin resonance spectral analysis was performed. Outer membrane preparations containing LPS with a long polysaccharide chain were much less fluid than preparations containing LPS with a short polysaccharide chain, and treatment with EDTA to reduce LPS content resulted in increased mobility. These results indicate that the polysaccharide moiety of LPS directly or indirectly causes the restricted mobility of the lipid hydrocarbon chains in the outer membrane.

II. Physiology and Genetics of an E. coli Strain Important in Studies of LPS and Membrane Assembly. E. coli O111 is of great interest both because it is the most common of the E. coli strains that are enteropathogenic for infants and because it has a very well-characterized LPS with a very long polysaccharide chain. For the latter reason it has been used for studies of

the outer membrane in several laboratories. Such work has been hindered because standard genetic manipulations have been heretofore impossible in this strain. We have worked out procedures for conjugation and have derived substrains which can be transduced. Transductions of loci at different map positions indicate that the positions are the same as for E. coli K-12. The methods devised will permit genetic analysis of this strain in as simple and complete a manner as E. coli K-12.

E. coli 0111 has been occasionally reported in the literature to carry a lysogenic phage, or a colicin, or both. We have now characterized a particle carried by our strain of E. coli 0111. This particle has the morphological characteristics, on electron microscopy, of the well-known transducing phage P1, but differs from it in antigenic specificity. It cannot plate well on any strain yet tested, but shows very high efficiency of generalized transduction into many strains, including E. coli K-12. It therefore manifests the characteristics of a defective transducing phage. It is of interest because it transduces a smaller portion of the genome than phage in current use and therefore may enable more precise fine-structure mapping.

Publications:

Bayer, M. E., and Leive, L.: The effect of ethylenediaminetetraacetate upon the surface appearance of Escherichia coli. J. Bacteriol., in press.

Coleman, W. G., Jr., Goebel, P. J., and Leive, L. Genetic analysis of Escherichia coli 0111:B4, a strain of medical and biochemical interest. J. Bacteriol. 130: 656-660, 1977.

Rottem, S., and Leive, L.: Effect of variations in lipopolysaccharide on the fluidity of the outer membrane of Escherichia coli. J. Biol. Chem. 252: 2077-2081, 1977.

| SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space) | U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT** | PROJECT NUMBER Z01 AM 24,120-06 LBP |
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PERIOD COVERED

July 1, 1976 through September 30, 1977

TITLE OF PROJECT (80 characters or less)

Studies in Traumatic Shock and Cellular Immunity

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

| | | | |
|---|---|---|---|
| PI: | Kehl Markley, M.D. | Medical Director, USPHS | LBP NIAMDD |
| OTHER: | Sanford M. Rosenthal, M.D. | Scientist Emeritus | LBP NIAMDD |

COOPERATING UNITS (if any)

None

LAB/BRANCH

Laboratory of Biochemical Pharmacology

SECTION

Section on Pharmacology

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NIAMDD, NIH, Bethesda, Maryland 20014

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| 3.1 | 1.3 | 1.8 |

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Previous studies from this laboratory have shown a defect in cellular immunity of burned mice with a decreased in vitro cytotoxicity of lymphocytes. In the present experiments, no defect was found in the sensitization of spleen cells from burned mice in the one-way mixed lymphocyte culture when assayed for cytotoxicity. In addition no inhibitor of cytotoxicity in the in vitro sensitization of spleen cells could be found in postburn serum.

Other experiments on the role of catecholamines in burn, tourniquet, and endotoxin shock demonstrated that propanolol significantly protected mice against death after burn and tourniquet trauma, while dibenamine significantly protected mice from endotoxin shock. In burn shock, measurements of the circulation indicated values closer to normal as early as 5 hours postburn in the propanolol pretreatment group than in controls. These results suggest that catecholamines which stimulate β receptors produce deleterious effects in traumatic shock.

In other experiments the swelling of ischemic trauma was largely controlled by hydrostatic forces, while in burn trauma osmotic forces played an important role. Application of a vacuum produced a paradoxical early decrease of swelling in burn, but not in tourniquet, trauma.

PHS-6040
(Rev. 10-76)

Project Description:

Objectives:

Our objectives are as follows: (I) to study the function of lymphocytes and macrophages in traumatic shock of mice; (II) to elucidate the mechanism of the defect in cellular immunity after burn injury; (III) to discover the mechanism of action of protective drugs in traumatic shock; (IV) to investigate the mechanisms involved in traumatic swelling; and (V) to study the nephrotoxic action of spermine.

Methods Employed:

(I) and (II). Mice were given a moderately severe burn of 2/3 body surface area in water at 70° for 7 seconds. Splenic lymphocytes from BALB/c ($H\text{-}2^d$) and C57 BL ($H\text{-}2^b$) mice were used in the one-way mixed lymphocyte culture. Stimulating cells were irradiated with 2,000 rads. A mixture of responding and stimulating cells at a ratio of 4 : 1 was incubated at 37° with 5% $CO_2$ for 5-6 days. The cells were harvested, and viable cells were counted using trypan blue exclusion. Then 1-ml of sensitized cells ($2 \times 10^6$) was added to 1 ml of $^{51}Cr$-labeled target cells ($5 \times 10^5$) and incubated for 4 hours at 37°. Cytotoxicity was measured by the amount of $^{51}Cr$ released from target cells into the supernatant.

(III). Burn trauma was produced in NIH female mice by the method described above, while tourniquet shock was produced by placing rubber bands in the inguinal region of both hindlegs for 90 minutes. Endotoxin shock was produced by intraperitoneal injection of 0.45 mg Escherichia coli lipopolysaccharide. A range of concentrations of the drugs epinephrine, norepinephrine, isoproterenol, dibenamine, and propanolol was injected intraperitoneally before or after the shock model studied. Measurements of bleeding volume, hematocrit, serum proteins, fluid lost into local damaged tissues, and core (colon) temperatures were made at frequent intervals before and after experimental shock. Mortality studies were performed to test the efficacy of various drugs in shock.

(IV). Swelling of the mouse tail was measured after burn and tourniquet trauma, and the influence of locally applied external pressure or a mild vacuum on swelling was studied.

(V). An investigation was made of antagonists to the nephrotoxic action of spermine using mortality as the assay.

Major Findings:

(I) and (II). In a series of 18 mixed lymphocyte culture experiments with splenic lymphocytes collected from BALB/c mice from 1-21 days postburn, the responder cells showed greater cytotoxicity than normal in 72% of the experiments ($P < 0.05$) and lesser cytotoxicity than normal in only 17% of the experiments ($P < 0.05$). In the same series, the stimulator cells showed normal

cytotoxicity in 58% of the experiments, with 18% higher and 24% lower. Addition of serum from burned mice collected from 1-42 hours postburn to the mixed lymphocyte culture showed no significant change in cytotoxicity over that produced by normal mouse serum. These results demonstrate: (i) that the necessary contingent of cell types required for generating a normal cytotoxic response in vitro is present in the spleens of burned mice and (ii) that no evidence could be found for a humoral inhibitor of sensitization for spleen cells in serum from burned mice.

(III). These studies demonstrated: (i) the intraperitoneal administration of epinephrine, norepinephrine, or isoproterenol 1/2 hour before burning had no significant effect on shock or 48 hour mortality; the same drugs in a nontoxic dose of $5 \times 10^{-4}$ mg/kg per mouse significantly increased burn shock mortality ($P < 0.05$). (ii) Pretreatment of mice with 25-50 mg/kg propanolol (a $\beta$ receptor-blocking drug) significantly decreased shock mortality after burn and tourniquet trauma ($P < 0.05$), whereas the $\alpha$ blocking drug (dibenamine) had no beneficial effect. (iii) Pretreatment of mice with the $\alpha$ receptor-blocking drug, dibenamine (25 mg/kg) significantly decreased shock mortality after endotoxin shock ($P < 0.05$). (iv) In measurements of the circulation, pretreatment of burn shock with propanolol produced a more normal bleeding volume, a lower hematocrit, and a lower serum protein level than controls as early as 5 hours postburn. After tourniquet shock, the propanolol-treated group showed no significant decrease in the loss of fluid into the injured area as compared with controls to account for the more normal bleeding volume observed in burn-shocked mice. (iv) The administration of catecholamines or $\alpha$ and $\beta$ receptor-blocking drugs had no significant effect on core temperature after burn or endotoxin shock during the acute or late periods, whereas the blocking drugs caused even lower core temperatures after tourniquet trauma.

(IV). The swelling of ischemic (tourniquet) trauma was largely controlled by hydrostatic forces, since swelling was completely suppressed by 60 mm mercury pressure. In burn trauma, osmotic forces were important because swelling occurred at the highest pressure used (120 mm Hg). Negative pressures caused corresponding increases in swelling in ischemic trauma, but with burns, a paradoxical decrease in early swelling was noted.

Significance to Biomedical Research and the Program of the Institute:

These studies aid in the understanding of the mechanism of impaired cellular immunity after trauma, clarify the role of catecholamines in the production of fatal shock after trauma and endotoxin, and elucidate the basic mechanisms of traumatic swelling.

Proposed Course:

(I) To study the function of lymphocytes and macrophages in cellular immunity after burn and tourniquet trauma. (II) To investigate the function of lymphocytes in gnotobiotic mice. (III) To study factors that influence phagocytosis and intracellular killing of bacteria in macrophages. (IV) To investigate biochemical mechanisms involved in swelling and in the basis of action of spermine.

Publications:

Markley, K., Smallman, E. T., and LaJohn, L. A.: The effect of thermal trauma in mice on cytotoxicity of lymphocytes. Proc. Soc. Exp. Biol. Med. 154: 72-77, 1977.

Markley, K., and Smallman, E. T.: Effect of thermal trauma on numbers and function of T and B cells from mouse spleen. Int. Arch. Allergy Appl. Immunol., in press.

| SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space) | U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT | PROJECT NUMBER Z01 AM 24,140-11 LBP |
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PERIOD COVERED
July 1, 1976 through September 30, 1977

TITLE OF PROJECT (80 characters or less)

Chemistry and Mechanism of Pyridoxal Phosphate Enzymes

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT


PI: Edith W. Miles, Ph.D. Research Chemist LBP NIAMDD

OTHER: William Higgins, Ph.D. Visiting Associate LBP NIAMDD


COOPERATING UNITS (if any)

None

LAB/BRANCH
Laboratory of Biochemical Pharmacology

SECTION
Section on Pharmacology

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20014

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| 2.2 | 2.0 | 0.2 |

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☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)


Studies of the tryptophan synthase $\alpha_2\beta_2$ complex serve as a model for studies of more complicated multienzyme complexes. Methods have been developed for the resolution of the holo $\alpha_2\beta_2$ complex and for the dissociation and reassociation of the $\alpha$ and $\beta_2$ subunits. Factors which influence subunit association and dissociation are being studied using the methods of gel filtration, difference absorbance, circular dichroism measurements, and enzymatic activity measurements. The results show that pyridoxal 5'-phosphate is tightly bound in the holo $\alpha_2\beta_2$ complex and can only be removed after addition of a salt which causes subunit dissociation. The finding that pyridoxal 5'-phosphate derivatives are much more strongly bound to the $\alpha_2\beta_2$ complex than to the $\beta_2$ subunit indicates that strong binding forces, in addition to the Schiff base linkage, exist in the $\alpha_2\beta_2$ complex but not in the $\beta_2$ subunit. The identification of active site residues and subunit interaction site residues is being investigated using chemical modification and a specific affinity label.





PHS-6040
(Rev. 10-76)

Project Description:

Objectives:

Studies of the tryptophan synthase $\alpha_2\beta_2$ complex of Escherichia coli are directed at elucidating how the $\alpha$ and $\beta_2$ subunits are regulated when they associate to form an $\alpha_2\beta_2$ complex. The work is designed to serve as a simple model to facilitate studies of the regulation of more complicated multienzyme complexes in higher organisms. Studies of tryptophan synthase take advantage of the fact that the cofactor of the $\beta_2$ subunit, pyridoxal 5'-phosphate, serves as an optical probe of the active site; this cofactor forms intermediate complexes with substrates and inhibitors which can be followed by optical methods. Experiments are designed to study the mechanism of the pyridoxal 5'-phosphate catalyzed reactions of the $\beta_2$ subunit and the effects of the $\alpha$ subunit on these reactions. Studies are also aimed at determining the essential catalytic residues in the active site region of the $\beta_2$ subunit and determining how the environment of these residues is changed by interaction with the $\alpha$ subunit. The results of these several approaches should give a picture of the combined active site and of the subunit interaction site in the $\alpha_2\beta_2$ complex.

Methods Employed:

Crystalline $\alpha$ and $\beta_2$ subunits and $\alpha_2\beta_2$ complex of tryptophan synthase are prepared and assayed by methods previously developed in this laboratory. Absorbance spectra and difference absorbance spectra are made with a Cary 118 spectrophotometer. Circular dichroism spectra and fluorescence spectra are also made. Subunit association studies are made using gel filtration. Chemical modification studies are carried out using diethylpyrocarbonate, 5,5'-dithio-bis-(2-nitrobenzoic acid), 2-nitro-5-thiocyanatobenzoic acid, iodoacetic acid, and butanedione. Affinity labeling is carried out using 4-bromoacetylpyridoxamine 5'-phosphate, which has been synthesized in our laboratory. Modified enzymes are examined for their ability to combine with pyridoxal 5'-phosphate or with $\alpha$ subunit and for their ability to carry out partial reactions. Modified amino acid residues and peptides are identified by labeling with radioactive compounds and by techniques of proteolytic digestions, peptide mapping, ion exchange chromatography, electrophoresis, gel filtration, disc gel electrophoresis, thin-layer chromatography, nuclear magnetic resonance, mass spectrometry, infrared spectrometry, and amino acid analysis.

Major Findings:

Pyridoxal 5'-phosphate derivatives are much more strongly bound to the $\alpha_2\beta_2$ complex than to the $\beta_2$ subunit. This finding indicates that strong binding forces, in addition to the Schiff base linkage, exist in the $\alpha_2\beta_2$ complex but not in the $\beta_2$ subunit. Resolution of the holo $\alpha_2\beta_2$ complex requires addition of a high concentration of a salt such as KSCN, which dissociates the subunits. Hydroxylamine greatly accelerates the rate of resolution of the holo $\alpha_2\beta_2$ complex in the presence but not in the absence of KSCN. Removal of pyridoxal 5'-phosphate results in decreased association between the $\alpha$ and $\beta_2$ subunits.

These results not only increase our understanding of pyridoxal 5'-phosphate binding and subunit association in the holo $\alpha_2\beta_2$ complex but also provide useful methods for resolving the holo $\alpha_2\beta_2$ complex and for separating the $\alpha$ and $\beta_2$ subunits. The separate subunits can thus be purified from a single strain of E. coli which produces the tryptophan synthase complex is very high yield. The apo $\alpha_2\beta_2$ complex obtained by this method is useful for studies of substances which affect subunit interaction. Certain phosphate-containing substrates and certain ions, including phosphate and sulfate, stimulate the association of the apo $\beta_2$ and $\alpha$ subunits to form an apo $\alpha_2\beta_2$ complex.

Essential sulfhydryl residues of the $\beta_2$ subunit are modified by 5,5'-dithiobis-(2-nitrobenzoic acid), 2-nitro-5-thiocyanatobenzoic acid), and by an affinity label synthesized by Dr. William Higgins: bromoacetylpyridoxamine 5'-phosphate. The identification of the modified residues and the sequence of the tryptic peptides containing these modified residues is in progress.

The results from several types of studies above suggest that the pyridoxal 5'-phosphate binding site and certain active site residues are buried in the tightly associated $\alpha_2\beta_2$ complex. Thus, the active site of the $\beta_2$ subunit may form part of the subunit interaction site in the $\alpha_2\beta_2$ complex.

Publications:

Miles, E. W.: Modification of histidyl residues in proteins by diethylpyrocarbonate. In Hirs, C. H. W., and Timasheff, S. N. (eds.): Methods in Enzymology, Enzyme Structure, Part E. New York, Academic Press, in press.

Miles, E. W., and Moriguchi, M.: Tryptophan synthase of Escherichia coli: removal of pyridoxal 5'-phosphate and separation of the $\alpha$ and $\beta_2$ subunits. J. Biol. Chem., in press.

| SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space) | U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT** | PROJECT NUMBER Z01 AM 24,260-11 LBP |
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PERIOD COVERED
July 1, 1976 through September 30, 1977

TITLE OF PROJECT (80 characters or less)

Enzymatic Studies of Nucleic Acid Metabolism

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT


| | | | |
|---|---|---|---|
| PI: | Nancy G. Nossal, Ph.D. | Research Chemist | LBP NIAMDD |
| OTHER: | E. Bruce Konrad, Ph.D. | Staff Fellow | LBP NIAMDD |


COOPERATING UNITS (if any)
Boris M. Peterlin, M.D., and Jacob V. Maizel, Ph.D., National Institute of Child Health and Human Development, National Institutes of Health

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Laboratory of Biochemical Pharmacology

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)


Replication of T4 DNA in vivo requires several proteins encoded by the phage DNA including the gene 43 DNA polymerase, the gene 32 DNA unwinding protein, the gene 44 and 62 DNA-dependent ATPase, the gene 45 protein, and the gene 41 single-stranded DNA-requiring nucleotidase. We have purified these proteins and have shown that they stimulate DNA synthesis in vitro with the T4 DNA polymerase with both native and single-stranded templates. We are presently studying the mechanisms by which chains are initiated with circular single-stranded templates with these proteins, as well as the mechanisms by which they accelerate the elongation of the primer and unwind duplex templates.

Using a new screening procedure, we have identified a temperature-sensitive E. coli mutant (mut3) with a high mutation frequency (mutator) which maps at the dnaE locus encoding DNA polymerase III and complements other dnaE mutants in vivo. DNA polymerase III activity from this mutant is not thermolabile in vitro, but the specific activity for polymerase III decreases after growth at nonpermissive temperatures. The increased mutation frequency (about 100-fold) appears to result from incorporation of incorrect nucleotides during DNA synthesis rather than to induction of the "error prone" repair system.

Project Description:

Objectives:

To determine the characteristics and functions of enzymes which are involved in the synthesis, modification, and degradation of DNA.

Major Findings and Proposed Course:

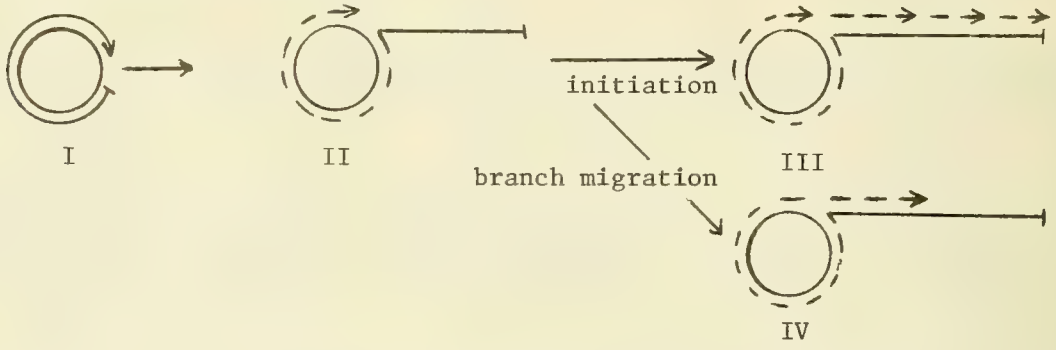
I. Proteins Required for T4 Bacteriophage DNA Synthesis. We are using E. coli infected with T4 bacteriophage as a system in which to identify and characterize the reactions catalyzed by enzymes required for DNA replication, since this phage appears to induce the synthesis of the enzymes required for its own DNA synthesis, rather than depending on host proteins. Phage with mutations in genes coding for many of these enzymes are available due to the work of Edgar, Epstein, and their collaborators, and phage-infected bacteria can be grown in quantities allowing purification of sufficient enzyme for biochemical and physical studies. The work has been facilitated by a regulatory phage mutant characterized by Wiberg which produces high levels of several of these enzymes.

Genetic studies by Edgar and Epstein et al. have shown that the proteins encoded by the phage genes 32, 41, 43, 44, 45, and 62 are required for DNA synthesis. Many other phage proteins are required for the synthesis of DNA precursors or are involved in controlling the time or extent of viral DNA synthesis. We have previously developed procedures for the purification of the gene 32 DNA unwinding protein; the gene 43 DNA polymerase which also has 3' to 5' exonuclease activity; the complex of the gene 44 and gene 62 proteins, which is a DNA-dependent ATPase (and dATPase); the gene 41 protein, a single-stranded DNA-requiring nucleotidase which hydrolyzes rGTP and rATP at similar rates and dGTP and dATP at about half this rate; and the gene 45 protein which stimulates the ATPase activity of the 44-62 protein. The gene 45 protein is also required for late mRNA transcription (Geiduschek) and binds specifically to the T4 phage-modified form of the host RNA polymerase (Ratner).

These six proteins are required for DNA synthesis in vitro with native duplex DNA such as T4 DNA or the closed circular DNA from the plasmid col E1. This synthesis requires rATP or a high concentration of dATP and under optimal conditions leads to product in excess of DNA used as template.

We have characterized the products by electron microscopy in collaboration with Boris M. Peterlin and Jacob V. Maizel (National Institute of Child Health and Human Development). Synthesis on a duplex template begins using the 3' OH group at a nick on the primer (I). The old strand complementary to the strand serving as template is displaced when new DNA is made. This leads to single-stranded branches or free single strands from linear duplex T4 DNA and duplex circles with very long single-stranded tails (rolling circles) from circular templates like col E1 (II). Later in the reaction there is extensive synthesis of DNA complementary to the displaced single-strand (III). Synthesis of the second strand presumably requires de novo initiation of a new chain, although we have not yet been able to show this directly or to rule out completely

the use of a complementary oligonucleotide as a primer. We also see structures like IV, in which the replication switches from one strand to the other by a process called branch migration.




Under the conditions used for replication, 32 protein binds to the supercoiled DNA of col E1, as shown by finding one bubble on 60% of the circles after fixing the protein to DNA with glutaraldehyde. Analysis of molecules fixed with 32 protein and then cut with the restriction nuclease ecoR1 showed that these loops occurred in one of two places, with one location predominating. The areas binding 32 protein corresponded to those most easily denatured by alkali. 32 Protein did not bind to relaxed (closed circles without supercoiling) or linear col E1 DNA molecules under replicating conditions, but did bind at low ionic strength with EDTA replacing $Mg^{2+}$.

In order to determine whether synthesis on duplex circles began preferentially at the areas binding 32 protein, we related the length of the displaced strand in replicating molecules to either the position cut by the ecoR1 nuclease or to bubbles formed by partial denaturation with alkali. Preliminary results suggest that synthesis does not originate preferentially at the 32 binding site or any other unique site. Synthesis can probably begin using a nick in any location, although the use of multiple specific sites has not yet been ruled out.

Synthesis on a duplex template requires unwinding of the original helix. This unwinding must involve one or more of the proteins whose function is still unknown (41, 44/62, or 45) since it is not catalyzed to a significant extent by the polymerase or 32 protein, separately or together. We have tried to determine the requirements for unwinding using centrifugal and microscopic assays. Synthesis and unwinding are closely coordinated since we find no evidence for unwinding in the absence of any of the proteins or nucleotides required for DNA synthesis.

During the next year we will try to determine whether the initiation of new chains on single-stranded templates occurs at specific sites. We will try to separate the synthetic reaction into stages in order to learn the function of the individual proteins.

II. Isolation of Mutants Controlling Mutation Frequencies in E. coli. The fidelity of replication of the DNA of E. coli is extremely high (about 1 error

per $10^9$ bases replicated). One might anticipate that the enzymes involved in E. coli replication could be damaged or modified through mutation in such a way as to lower the fidelity of the replication. Such "mutator mutations" would be useful in determining which enzymes are involved in maintaining the fidelity of DNA replication.

We have developed a method which permits large numbers of mutagenized colonies to be screened for the mutator trait and have used this to select a much larger number (1,500) of mutator mutations than had previously been isolated by other workers. Only one of these mutants (mut3) was a temperature-sensitive, conditionally-lethal mutation, indicating that it affected an essential function. Mut3 appears to be in the structural gene (dnaE) for DNA polymerase III, the polymerase required for DNA replication, since it maps at the same position as previously isolated mutants in this gene and complements dnaE mutants in vivo. DNA polymerase III activity was not thermolabile in partially purified preparations from mut3. However, the specific activity for polymerase III decreased after shifting mut3 to a nonpermissive temperature, suggesting that either the enzyme is thermolabile in vivo or that its synthesis is thermosensitive. In vivo, DNA synthesis shuts off gradually at high temperatures, in contrast to the more rapid shutoff of other mutants in dnaE.

Mut3 enhances mutation frequencies about 100-fold. This increase is probably due to the incorporation of incorrect nucleotides during DNA synthesis, since we have shown that it does not depend on the induction of the "error prone" repair system.

Our failure to isolate mutants other than mut3, which are mutators due to alterations in DNA replication enzymes, indicates such mutants are rare, or do not exist. One interpretation of this finding is that there are efficient mechanisms which correct most of the errors made during replication.

Publications:

Gillin, F. D., and Nossal, N. G.: Control of mutation frequency by bacteriophage T4 DNA polymerase. I. The CB120 antimutator DNA polymerase is defective in strand displacement. J. Biol. Chem. 251: 5219-5224, 1976.

Gillin, F. D., and Nossal, N. G.: Control of mutation frequency by bacteriophage T4 DNA polymerase. II. Accuracy of nucleotide selection by the L88 mutator, CB120 antimutator, and wild type phage T4 DNA polymerases. J. Biol. Chem. 251: 5225-5232, 1976.

Project No. ZO1 AM 24,510-02 LBP
1. Biochemical Pharmacology
2. Pharmacology
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1976 through September 30, 1977

Project Title: Phosphatidylserine Synthetase Mutants of Escherichia coli

Previous Serial Number: Same

Principal Investigator: Christian R. H. Raetz, M.D., Ph.D.
Research Associate in Pharmacology, NIGMS
Guest Worker, LBP, NIAMDD

Other Investigators: None

Cooperating Units: None

Man Years: None

Project Description: This project was terminated on June 30, 1976.

Publications:

Raetz, C. R. H.: Phosphatidylserine synthetase mutants of Escherichia coli: genetic mapping and membrane phospholipid composition. J. Biol. Chem. 251: 3242-3249, 1976.

Wickner, S. H., Wickner, R. B., and Raetz, C. R. H.: Overproduction of dna gene products by Escherichia coli strains carrying hybrid ColE1 plasmids. Biochem. Biophys. Res. Commun. 70: 389-396, 1976.

| SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space) | U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT** | PROJECT NUMBER Z01 AM 24,710-27 LBP |
|---|---|---|

PERIOD COVERED
July 1, 1976 through September 30, 1977

TITLE OF PROJECT (80 characters or less)

Estimation, Metabolism, and Function of Amines

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

| | | | |
|---|---|---|---|
| PI: | Herbert Tabor, M.D. | Medical Director, USPHS | LBP NIAMDD |
| | and Chief, Laboratory of Biochemical Pharmacology, | | LBP NIAMDD |
| | and Chief, Section on Pharmacology | | LBP NIAMDD |
| | Celia White Tabor, M.D. | Medical Director, USPHS | LBP NIAMDD |
| OTHERS: | Edmund W. Hafner, Ph.D. | Staff Fellow | LBP NIAMDD |
| | Murray S. Cohn, Ph.D. | Staff Fellow | LBP NIAMDD |
| | Achilles A. Demetriou, M.D. | Guest Worker | LBP NIAMDD |

COOPERATING UNITS (if any)

None

LAB/BRANCH
Laboratory of Biochemical Pharmacology

SECTION
Section on Pharmacology

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20014

| TOTAL MANYEARS: | PROFESSIONAL: | OTHER: |
|---|---|---|
| 6.5 | 5.0 | 1.5 |

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

We are continuing our studies on the biological role of polyamines. Particular attention is being directed towards the selection of Escherichia coli and yeast mutants that are defective in the biosynthesis of these amines, and a study of the resultant phenotypic effects.

Studies are also being carried out on the characterization of the specific enzymes involved in spermidine biosynthesis, with particular emphasis on adenosylmethionine decarboxylase from prokaryote and eukaryote sources.



PHS-6040

Project Description:

Mutations in Spermidine Biosynthesis in Escherichia coli and Saccharomyces cerevisiae.

New methods have been developed for the detection of bacterial mutants defective in reactions producing $CO_2$. Although these methods were developed for detection of adenosylmethionine decarboxylase-deficient mutants, they will be of use in finding any mutants where no easy selection system is available.

Two general methods have also been developed for markedly increasing the frequency with which Mu-insertion mutations are obtained.

With these methods, mutations have been obtained in the various enzymes involved in spermidine biosynthesis (e.g., ornithine decarboxylase, arginine decarboxylase, agmatine ureohydrolase, and adenosylmethionine decarboxylase). Extensive mapping and analytical studies have been carried out on the adenosylmethionine decarboxylase-deficient mutant (called speD). The map position is 2.7 minutes on the E. coli chromosome. No adenosylmethionine decarboxylase or spermidine is present in the Mu-insertion mutants. Despite this complete lack of spermidine, only a small decrease is observed in the growth rate of the mutant.

A comparable mutant has also been obtained in S. cerevisiae. No adenosylmethionine decarboxylase or spermidine is present. The growth rate is only slightly decreased. Extensive mapping experiments have been carried out.

Adenosylmethionine decarboxylase has been purified to homogeneity from yeast and shown to contain one pyruvate per subunit, covalently bound, presumably at the active site. This is the first time this unusual prosthetic group has been found in eukaryotes.

Adenosylmethionine decarboxylase has also been purified to homogeneity from rat liver and shown also to contain one pyruvate per subunit.

Further work has been carried out on our methods, using the amino acid analyzer, for the polyamines and for glutathione and its derivatives. A particularly useful byproduct of these studies has been the development of a specific assay for carboxyglutamate (the amino acid recently described in prothrombin).

Publications:

Tabor, C. W., and Tabor, H.: An automated ion-exchange assay for glutathionine, Anal. Biochem. 78: 543-553, 1977.

Tabor, C. W., and Tabor, H.: 1,4-Diaminobutane (putrescine), spermidine, and spermine. Annu. Rev. Biochem. 45: 285-306, 1976.

Tabor, H., Hafner, E. W., and Tabor, C. W.: Localized mutagenesis of any specific region of the bacterial chromosome with phage Mu. J. Bacteriol., in press.

Tabor, H., and Tabor, C. W.: An automated assay of γ-carboxyglutamate with an anion-exchange column. Anal. Biochem. 78: 554-556, 1977.

Tabor, H., Tabor, C. W., and Hafner, E. W.: Convenient method for detecting $^{14}CO_2$ in multiple samples: application to rapid screening for mutants. J. Bacteriol. 128; 485-486, 1976.

Honors and Awards:

Dr. Herbert Tabor was elected a member of the National Academy of Sciences of the United States of America on April 26, 1977.

| SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space) | U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT** | PROJECT NUMBER Z01 AM 24,940-04 LBP |
|---|---|---|

PERIOD COVERED
July 1, 1976 through September 30, 1977

TITLE OF PROJECT (80 characters or less)

Mechanisms of Inheritance in Saccharomyces cerevisiae

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

| | | | |
|---|---|---|---|
| PI: | Reed B. Wickner, M.D. | Senior Surgeon, USPHS | LBP NIAMDD |
| OTHERS: | Michael J. Leibowitz, M.D., Ph.D. | Senior Staff Fellow | LBP NIAMDD |
| | Akio Toh-e, Ph.D. | Visiting Associate | LBP NIAMDD |

COOPERATING UNITS (if any)

None

LAB/BRANCH
Laboratory of Biochemical Pharmacology

SECTION
Section on Pharmacology

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20014

| TOTAL MANYEARS: | PROFESSIONAL: | OTHER: |
|---|---|---|
| 2.5 | 2.2 | 0.3 |

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The presence of a 1.4 x $10^6$ dalton double-stranded RNA containing virus-like particles (the killer plasmid or killer virus) in Saccharomyces cerevisiae enables cells to secrete a protein toxin and be immune to this toxin. We have defined 24 chromosomal genes (called mak1, . . .) needed to replicate or maintain the killer plasmid. Methods for complementation and genetic mapping of these genes have been devised. A nonprotein mitochondrial gene product inhibits killer plasmid replication. This effect is prevented by the mak10 product. One dominant chromosomal mutation bypasses the plasmid's need for two mak genes, while another dominant chromosomal mutation bypasses the plasmid's need for five other mak genes. One chromosomal gene is needed to maintain the killer plasmid, to maintain mitochondrial DNA, and for mitotic cell growth. Chromosomal mutations resulting in increased production of killer toxin (superkillers) have also been observed.

Project Description:

A species of double-stranded (ds) RNA (called M) of 1.4 x $10^6$ daltons makes yeast cells carrying it (called killers) able to secrete a protein toxin which kills cells (called sensitives) not carrying this ds RNA species. M ds RNA is found in the cell encapsulated in virus-like particles. Infection by these virus-like particles has not been demonstrated. Thus, the killer phenotype is determined by M ds RNA which is genetically a plasma (the killer plasmid) but physically a virus. M ds RNA is transmitted in matings.

Mak Genes: Complementation and Genetic Mapping. We have isolated a large number of mutants in chromosomal genes (called mak genes) needed to maintain the killer plasmid (virus). We have developed a method for performing genetic complementation tests between different mak mutants. This has allowed us to determine that these mutants are located in 24 different mak genes. We have now located 15 of these genes on the genetic map of yeast. Genetic mapping in yeast is a rather laborious procedure, and we have developed a new, quicker mapping method, employing aneuploidy and a specially constructed triploid strain. This method simplifies mapping any yeast gene and can be used in any other organism where tetrad analysis is possible.

Other Functions of Mak Genes. Several mak genes are involved in processes other than replication of the killer ds RNA plasmid (virus). This year we have studied one such gene, called pets, in some detail. We find that the pets gene is required for (i) maintenance of the killer plasmid, so it is a mak gene; (ii) maintenance of mitochondrial DNA; and (iii) mitotic growth of yeast cells. Pets is the first chromosomal gene shown to be essential for replication of the mitochondrial genome. Pets is not needed, however, for replication of four other plasmids of yeast. We have not found what the role of pets is in cellular growth, but it is essential. Mutations in several other mak genes result in decreased growth rates. They may indeed be essential genes. Collection of several mutants in each mak gene will be necessary to resolve this question.

Mutations Which Bypass Mak Genes. We have isolated mak bypass mutants in which mutation of another gene has bypassed one or more mak genes. One mak gene (mak10) is bypassed by mutation of the mitochondrial genome. Thus, in the absence of a mitochondrial gene product, the mak10 gene is no longer needed for killer plasmid (virus) replication. This mitochondrial product is not a protein. The mitochondria codes for a large number of tRNAs and the rRNAs. We are now attempting to locate the gene of interest on the yeast mitochondrial genetic map. We propose that a mitochondrial RNA product inhibits killer plasmid replication and that the mak10 product prevents this inhibition.

We have found dominant chromosomal mutations which bypass mak7 mutations and the mak defect of pets mutants. These mutations define a new chromosome on the yeast map and also have interesting genetic properties. The mutations arise at a high frequency and revert to the wild type state also at a very high frequency.

We have found a third mutation, which bypasses the need for five mak genes (of the ten so far tested). This mutation is a dominant chromosomal mutation

in a locus distinct from that discussed in the preceding paragraph. The bypass of half of the mak genes tested suggests that in this case a substantial change in the mode of replication of the killer plasmid (virus) occurs in these mutants.

The study of bypass mutants such as those described here is of value in three ways: (i) It provides a functional classification of the large number of mak genes. We presume that mak genes bypassed by the same mutation must be functionally related in some way. (ii) It promises to shed light on the specific functions of the mak genes bypassed. (iii) The gene in which the bypass mutation occurs must be involved in some way in killer plasmid replication.

Superkiller Mutants. Starting with a wild type killer strain, we have isolated mutants which produce more than the normal amount of toxin activity (called superkillers). These mutants are located in six unlinked chromosomal genes. In each case, the superkiller (mutant) phenotype is recessive to the wild type. The ds RNA of one such mutant has now been examined, and there is an apparent marked increase in the amount of the M ds RNA, i.e., the killer plasmid is overproduced. These genes may be regulators of the number of plasmid copies per cell.

RNA Polymerase Activity. We have discovered an RNA polymerase activity in virus-like particles isolated from killer strains of yeast. The product of this enzyme is now under study. It is possible that this represents a transcriptase or replicase activity.

Publications:

Leibowitz, M. J., and Wickner, R. B.: A chromosomal gene required for killer plasmid expression, mating, and sporulation in Saccharomyces cerevisiae. Proc. Natl. Acad. Sci. U.S.A. 73: 2061-2065, 1976.

Wickner, R. B.: Deletion of mitochondrial DNA bypasses a chromosomal gene needed for maintenance of the killer double-stranded RNA plasmid (virus) of Saccharomyces cerevisiae. Genetics, in press.

Wickner, R. B.: Fungal killer factors. McGraw-Hill Yearbook of Science and Technology, in press.

Wickner, R. B.: Killer of Saccharomyces cerevisiae: a double-stranded ribonucleic acid plasmid. Bacteriol. Rev. 40: 757-773, 1976.

Wickner, R. B.: Overproduction of killer plasmid ds RNA in yeast: bypass of a chromosomal gene for ds RNA plasmid maintenance by deletion of mitochondrial DNA. Microbiology, 1978, in press.

Wickner, R. B., and Leibowitz, M. J.: Chromosomal genes essential for replication of a double-stranded RNA plasmid of Saccharomyces cerevisiae: the killer character of yeast. J. Mol. Biol. 105: 427-443, 1976.

Wickner, R. B., and Leibowitz, M. J.: Dominant chromosomal mutation bypassing chromosomal genes normally needed for killer double-stranded RNA plasmid replication in yeast. Genetics, in press.

Wickner, R. B., and Leibowitz, M. J.: Genetics of a double-stranded RNA plasmid: the killer character of Saccharomyces cerevisiae. Microbiology, 1977, pp. 571-575.

ANNUAL REPORT OF THE LABORATORY OF CHEMICAL PHYSICS,
NATIONAL INSTITUTE OF ARTHRITIS, METABOLISM AND DIGESTIVE DISEASES

The electronic structure and the molecular architecture of macromolecules continues to be one of the major foci of research in this Laboratory. This year the emphasis has shifted slightly in each of three major projects. The experimental electron spin resonance work on the heme iron(III) in cytochrome P450 and related model compounds has now been supplemented by a theoretical investigation by Kon, Rispin and Sato. In a continuation of the experimental investigations, Kon and Sato [with D. W. Nebert (CH-DPB)] have found a way of using the temperature dependence of the ESR results to study in vivo, as well as in vitro, the relative amounts of high and low spin components of cytochrome P450.

Both the kinetic and thermodynamic studies of deoxyhemoglobin S gelation have been continued by Eaton with the collaboration of Hofrichter, Ross and Sunshine. But while the theoretical analysis has previously been confined by the necessity for assuming ideality of the hemoglobin solutions (activity directly proportional to concentration), the availability of additional experimental data has made it possible to incorporate solution non-ideality in the calculation of the enthalpy changes which occur in the polymerization and gelation of deoxyhemoglobin S. At physiological temperatures, the reaction is virtually entirely entropy driven with $\Delta S^\circ$ = 9.6 e.u. These more detailed studies are being used to model the molecular mechanism of the gelation with the hope that the additional understanding of the mechanism will provide a scientific rationale for the development of therapeutic measures for sickle cell disease. Eaton and Ferrone also have underway a new project using laser-induced photodissociation of carbomonoxyhemoglobin to investigate the rate of polymerization and deoxyhemoglobin S.

Hofrichter has used light-scattering to observe changes in the molecular size occurring during the previously observed delay period of deoxyhemoglobin S gelation. The reaction appears to follow a purely autocatalytic time course through the first three decades of detectable signal in the laser light scattering photometer constructed for this investigation. In still another study of the polymerization of sickle cell hemoglobin, Hofrichter has demonstrated, using linear dichroism on the aligned polymer phase of deoxyhemoglobin S, that carbomonoxyhemoglobin is excluded from the polymer phase even if it is only partially liganded.

Charney is applying electric dichroism measurements of polynucleotides to study the properties of polyelectrolytes by examining the relation between theories of the charge density of polyelectrolyte and their orientation in an electric field. These studies are still in an early stage, but it is clear that the orientation is very much dependent on the extent of counter-ion binding and that this in turn is dependent, in the case of polynucleotides, on the density of otherwise uncompensated phosphate charges, and so on the structure. Charney and Chen have also completed the electric-dichroism phase of a study of the structure of poly (C) and

are engaged in a study of the nature of double strand complexing of polynucleotides in polydispersed systems, where it appears that the formation of complete double strands is limited by the availability of short polymer strands. The process is driven to achieve the maximum complexing, rather than the maximum length of double strands.

The structure, conformation and molecular motions in phospholipids and model membrane systems are being investigated by Levin and his collaborators using vibrational spectroscopic methods (infrared and Raman). From a study of the temperature dependence of the Raman spectra of the gel phases of several phosphatidylcholines, Levin and Yellin have found characteristic temperatures for onset of hydrocarbon chain trans-gauche isomerization and have interpreted differences in terms of inter-chain interactions related to their orientations. Meanwhile, Bunow and Levin have used both normal and deuterated phospholipids to verify assignments of vibrational modes in the C-H (and C-D) stretching region, which give rise to intense Raman bands that are used to probe structural reorganizations in bilayers.

Another approach to conformation was pursued by Govil, who showed by $^{1}H$ and $^{13}C$ NMR that both phospholipids and triglycerides in $CDCl_3$ solution show rapid equilibration between the two forms known from X-ray crystallography and from earlier theoretical studies of the potential energy of lipid conformations.

Spectroscopic and quantum theoretical studies of small molecules provide another important facet of the Laboratory's program. Ultraviolet and vacuum ultraviolet studies by McDiarmid aimed at elucidating the nature of the Si-S bond have included investigation of the absorption spectra of $(SiH_3)_2S$, $[Si(CH_3)_3]_2S$, $(GeH_3)_2S$, $[Si(CH_3)_3]_2O$ and $[G(CH_3)_2]S$. In a continuing study of Rydberg transitions, the ultraviolet spectra of trans-CHF=CHF and trans-CDF=CDF were also measured and analyzed.

Charney and Rosenfield, continuing their study of chiral activity by quantum mechanical calculations (CNDO/S), found that the chiral activity of a dissymmetric diene is not only sensitive to substituents allylic to the end carbons, but that in addition, if the substituent is a methyl group, the activity is highly sensitive to the rotamer distribution. They were able to explain semiquantitatively the optical activity of $\alpha$-phellandrene on this basis. Rosenfield also extended her investigation of the magnetic circular dichroism (MCD) of benzene to the second excited state, correctly predicting the ordinary absorption and predicting the vibronic structure of the MCD of this band. Verification of this latter prediction awaits a high resolution experimental study of the MCD.

Using an extended Hückel method developed by Gouterman and his coworkers at the University of Seattle, Hanson and Eaton have calculated the spectra of imidazole-Fe-$O_2$-porphin, imidazole-Fe-CO-porphin, and imidazole-Fe-porphin complexes. The results have been used to assign the low energy transition in oxy-, deoxy-, and carbomonoxyhemoglobin.

Fisk, Miles and Becker (in collaboration with Pinnavaia at Michigan State University) have continued their study of the self-association of guanosine-5'-phosphate in neutral solution. From $^{13}C$ NMR relaxation times they found that at least two ordered structures are present with rotational correlation times of about $10^{-8}$ sec. They also discovered by $^{1}H$ NMR that the association process is highly dependent on cations that are present. Another metal ion interaction, $Li^{+}$ plus, N,N-dimethylformamide (DMF), was investigated by Rao and Becker, who found from NMR measurements that complexation of $Li^{+}$ increases the barrier to internal rotation in DMF by 3.5 kcal/mole.

Three-dimensional conformations of atoms in a number of biologically interesting compounds have been studied by the theoretical technique of computerized energy minimization. For example, Sharpless has predicted the molecular architecture of retinaldehyde derivatives important in vision and of DDT compounds, while Govil has found that in N-phenylmaleimide the two rings are inclined at an angle of about 25°, in accord with one of two structures compatible with NMR results.

Elucidation of the chemical and physical steps resulting from the absorption of light by a photoreceptor is an important goal in the Laboratory. Hagins and Yoshikami's studies of photon detection in vertebrate rods and cones have provided new evidence that $Ca^{++}$ ions released in the cells are an essential step in visual excitation. They have introduced a calcium-sensitive azo dye into the retinal cells by the phospholipid vesicle fusion method in order to monitor $Ca^{++}$ behavior. Meanwhile, Robinson and Hagins have discovered a new enzyme, a guanosine triphosphatase, which is capable of providing free energy for the excitatory cycle in rods. Although inactive in darkness, it is rapidly activated by low light exposures. Another new technique, a method for studying by high speed cinematography the fast freezing of biological samples for biochemical and electron microprobe analysis has been developed by Meisner and Hagins for application in the studies of visual excitation. Adams, also investigating some aspects of visual excitation, has explored possible complexes of retinal which could simulate the spectral characteristics of the visual pigment rhodopsin.

Shean has discovered that an artificial mosaic membrane, consisting of a liquid anion exchanging membrane in series with a high resistance porous cation exchanging membrane, can be selectively anion permeable for transport purposes, while showing cation permeability for electrical processes.

For a variety of studies in pharmacology and molecular biology it is desirable to have both enantiomers of an asymmetric compound in order to distinguish between specific and non-specific effects. Ziffer and Imuta have extended their studies on asymmetric synthesis to the preparation of alcohols of high optical purity by reduction of a series of benz-α-tetralone derivatives. They also prepared optically pure threo diols by reduction of a series of benzil derivatives and studied their further stereochemical reactions.

Weiss and Bhatnagar have prepared 2-fluoromorphine in order to study its analgesic action and receptor-binding ability in comparison with those of the 1-fluoro isomer. They have also elucidated the structure of a long-known anomalous demethylation product of 5-benzyldihydromorphineone and have explained the color dimorphism of 14-hydroxymorphinone in terms of differences in intermolecular hydrogen bonding in the two crystal forms.

Subjective symptoms such as irritation of the eyes and respiratory tract due to atmospheric pollutants have received wide publicity. Little is known of the systemic effects of ambient cigarette smoke. Altland and Parker have now shown that concentrations of suspended particulate matter (SPM) of 15 $mg/m^3$ and 13 ppm carbon monoxide (CO), derived from burning cigarettes, induced hypothermia in rats undergoing mild exercise. Also, SPM and CO concentrations of 34 $mg/m^3$ and 51 ppm respectively, produced elevations in the concentration of plasma lactic dehydrogenase in exercised rats. Higher smoke levels produced changes in the concentrations of plasma corticosterone creatine kinase and lactic dehydrogenase in rats at rest. However, the amount of cigarette smoke which produced systemic changes severe enough to result in changes in cellular membrane permeability greatly exceeded National Air Quality Standards for man (SPM 0.075 $mg/m^3$ and CO 9 ppm).

Much of the Laboratory's research is highly dependent on maintaining and extending sophisticated instrumentation and techniques. For example, the capabilities of our NMR center were improved with the construction of new probes for the study of $^{31}P$ and $^{113}Cd$ by Shindo and Tschudin, and the development of a microprocessor-based control and data acquisition system by Ader. In general, computers play an increasingly important role in many of our experiments. Jennings has maintained and expanded the program library for the heavily used time-sharing computer that is interfaced to a number of instruments in LCP and LMB.

Because of the heavy use of most of our NMR spectrometers, we are devoting considerable effort to improving the efficiency of their use. Weiss, Ferretti and Becker have made a careful theoretical analysis of the optimum strategies for measuring NMR relaxation times, since this is one of the most time-consuming of NMR experiments.

| SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space) | U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT** | PROJECT NUMBER Z01-AM-29000-05-LCP |
|---|---|---|

PERIOD COVERED
July 1, 1976 through September 30, 1977

TITLE OF PROJECT (80 characters or less)

Nuclear magnetic resonance: New methods and molecular structure determination

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

| | | | |
|---|---|---|---|
| PI: | Edwin D. Becker | Research Chemist | A LCP |
| OTHER: | Robert B. Bradley | Physicist | A LCP |
| | Rolf G. Tschudin | Electronic Engineer | A LCP |
| | Girjesh Govil | Visiting Associate | A LCP |
| | Cherie L. Fisk | Staff Fellow | A LCP |
| | Linda Sweeting | Guest Worker | A LCP |

COOPERATING UNITS (if any)

H. T. Miles (NIAMDD-LMB)
J. A. Ferretti, G. Weiss (DCRT)
R. K. Gupta, Institute for Cancer Research
T. J. Pinnavaia, Michigan State University
C.N.R. Rao (PL-480 project, Indian Institute of Technology, Kanpur)

LAB/BRANCH
Laboratory of Chemical Physics

SECTION
Section on Molecular Biophysics

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20014

| TOTAL MANYEARS: | PROFESSIONAL: | OTHER: |
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| 6 | 5 | 1 |

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

NMR studies of nucleotide conformation and nucleotide interactions concentrated on the elucidation of structure of the associated form of guanosine-5'-phosphate at neutral pH. $^{13}C$ relaxation time measurements provided limits on the size of the aggregate, while $^{1}H$ spectra showed that the association is sensitive to the cations present.

From dynamic NMR studies we have found that binding of $Li^{+}$ to N,N-dimethylformamide increases the barrier to internal rotation by 3.5 Kcal/mole.

$^{1}H$ and $^{13}C$ NMR studies of phospholipids and triglycerides in $CDCl_3$ solution show the existence of an equilibrium between the two forms known from crystallographic work.



PHS-6040
(Rev. 10-76)

Project No.: ZO1-AM-29000-05-LCP

Projection Description:

Objectives:

1. Elucidation of the forces within and between molecules, especially those of potential biological importance.
2. Development of spectroscopic methods for studying molecular structure and analyzing materials of chemical and biological interest.

Major Findings and Future Plans:

Studies on the structure of self-assembled sodium guanosine-5'-phosphate (GMP) in aqueous solution have been extended to include $^{13}C$ NMR relaxation measurements and an investigation of the structure-forming role played by alkali metal ions. Measurements of the spin-lattice relaxation time of specific carbons in the self-associated form of $Na_2$-GMP indicate that more than one type of regular ordered structure is present. In addition, the measurements provide a confirmation of our earlier estimate for the rotational correlation time of the largest species, ca. $10^{-8}$ sec. We have found using proton NMR that the existence and nature of the self-assembled GMP complex depends upon the alkali metal counterion. $K_2$GMP, $Rb_2$GMP and $Cs_2$GMP all form self-assembled complexes which differ among themselves, and differ from $Na_2$GMP, in the number of chemically similar but magnetically non-equivalent proton lines and in their respective melting profiles. $Li_2$GMP, $Tl_2$GMP and $(NMe_4)_2$GMP do not form detectable self-structures. These observations are unprecedented in nucleic acid chemistry and experiments planned for the coming year promise to further clarify the role of the alkali metal ion in nucleotides and related biological systems. (Fisk, Miles, Pinnavaia, Becker)

We have obtained $^1H$ and $^{13}C$ NMR spectra of two classes of lipids (triglycerides and phospholipids) in $CDCl_3$ solutions, which show that the observed $^1H$-$^1H$ and $^1H$-$^{13}C$ coupling-constants for both classes are very similar. The experimental data cannot be reconciled with the presence of either the pure "A" or pure "B" structure known from crystallography, which differ in gauche-trans isomerization. In both cases, the data indicate an equilibrium A $\rightleftharpoons$ B with population distribution of about 35:65. Since both A and B can be packed in lipid bilayers, it appears that such an equilibrium may also be present in biomembranes. (Govil)

Substituted maleimides have found several biological and technical applications. The conformational structure of N-phenyl maleimide has been investigated by molecular orbital methods (CNDO and PCILO). It is found that the most stable structure for this molecule is such that the planes passing through the two rings are inclined at an angle of about 25°. The results are in agreement with one of the two possible solutions obtained from the analysis of NMR spectrum of the molecule oriented in nematic solvent. (Govil, Khetrapal)

The structure of Poly (8BrA), an all syn polynucleotide, has been studied by NMR and potential energy minimization. Details are in report No. ZO1-AM-35000-13-LMB. (Miles, Govil, Fisk)

The measurement of spin-lattice relaxation times is an often rewarding, but time-consuming, NMR experiment. We have explored the strategy for carrying out such measurements in a manner designed to provide the maximum precision in the minimum amount of experimental time. Details of the work are reported in ZO1-CT-00025-01-DCRT. This work is now being extended to include the effects of common systematic errors. (Becker, Weiss, Ferretti, Gupta)

The effect of $Li^+$ ion binding to dimethylformamide has been studied by $^1H$ NMR. The barrier to internal rotation of the $N(CH_3)_2$ group was found to increase by 3.5 kcal/mole on binding of lithium ion. (Rao, Becker)

Our research program includes a significant segment devoted to the development of improved spectroscopic methods. During the last year our NMR laboratory has been upgraded by the addition of probes designed for the study of $^{113}Cd$ and $^{31}P$ with the NIH-270 NMR spectrometer. In addition, our CPS-2 pulse spectrometer has been fitted with a microprocessor-based control and data acquisition system. One or more multi-nuclear probes will soon be developed for the NIH-270, and the associated computer system will be improved to increase productivity on this fully utilized instrument. (Shindo, Tschudin, Ader, Becker)

Publications:

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Reported in Press - 1976:

Conformational Structure of Polynucleotides around the O-P bonds: Refined Parameters for CPF Calculations. Govil, G., Biopolymers 15, 2303 (1976).

| SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space) | U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT** | PROJECT NUMBER Z01-AM-29001-05-LCP |
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PERIOD COVERED
July 1, 1976 through September 30, 1977

TITLE OF PROJECT (80 characters or less)

Molecular Dynamics and Vibrational Characteristics of Membrane Assemblies

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

| | | | |
|---|---|---|---|
| PI: | Ira W. Levin | Research Chemist | A LCP |
| OTHER: | Ian R. Hill | Visiting Fellow | A LCP |
| | Margaret R. Bunow | NIH Research Fellow | A LCP |

COOPERATING UNITS (if any)

S. Abramowitz, National Bureau of Standards
I. Asher, Food and Drug Administration

LAB/BRANCH
Laboratory of Chemical Physics

SECTION
Section on Molecular Biophysics

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20014

| TOTAL MANYEARS: | PROFESSIONAL: | OTHER: |
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| 3.0 | 3.0 | |

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Vibrational Raman spectroscopic transitions are used to probe the dynamical processes involved in lipid-lipid interactions in model membrane bilayer assemblies. Specifically, spectral changes are used to monitor integral and peripheral membrane components, to determine intrachain and lateral intermolecular disordering effects within the phospholipid matrix, to estimate the size of the cooperative unit involved in the gel-liquid crystalline phase transition and to describe the formation of membrane complexes.



PHS-6040
(Rev. 10-76)

Project Description:

Within the project area of the vibrational dynamics of membrane components and membrane assemblies our objectives are to probe, clarify and interpret the vibrational spectroscopic characteristics of biological membranes and related systems. The general approaches include: (a) analyses of the Raman and infrared spectra of selected systems, (b) determination of reliable intramolecular force fields when applicable, and (c) interpretation of molecular charge distribution (infrared and Raman intensity) parameters.

A variety of laser Raman and infrared spectroscopic procedures provide the required data for the various vibrational analyses.

The studies during the part year dealt specifically with pure lipid components and with a variety of model biological bilayer systems. These results are described in the following paragraphs.

(1) Vibrational Raman spectra of multilayers of 1,2-dimyristoyl, 1,2-dipalmitoyl and 1,2-distearoyl phosphatidylcholine were recorded as a function of temperature for the gel phase from -180°C to values slightly below the pretransition temperatures. Temperature profiles for band intensity ratios involving the acyl chain C-C stretching modes define a characteristic temperature $T_g$ which denotes the onset of hydrocarbon chain trans-gauche isomerization in the gel. $T_g$ for the dimyristoyl ($C_{14}$ chain lengths), dipalmitoyl ($C_{16}$ chain lengths) and distearoyl ($C_{18}$ chain lengths) systems are approximately -40°, -40° and 5°C, respectively. The higher $T_g$ value for distearoyl phosphatidylcholine is associated with increasing interactions between the terminal chain areas of the individual monolayers forming the bilayer unit. This increased interaction between bilayer halves is presumably a consequence of the larger angle through which the hydrocarbon chains are tilted in the $C_{18}$ phospholipid gel.

Temperature dependent peak height intensities for the 3000-2800 C-H stretching region provide convenient probes for quantitatively describing the increase in lateral chain packing disorder as the gel assemblies are warmed. The temperature profiles for various methylene mode peak height ratios indicate that both the inter- (lateral packing) and intramolecular (trans-gauche) disordering processes are related and are initiated at nearly the same temperatures for each lipid system. Increases in the van't Hoff enthalpies which result from the disordering of the lipid gel matrix by a lattice expansion were estimated from the Raman spectra of the $C_{14}$, $C_{16}$ and $C_{18}$ phospholipids to be approximately 1.5, 1.2 and 3.2 kcal/mol, respectively. (Levin and Yellin)

(2) The temperature dependence of the Raman spectral transitions assigned to the acyl chain C-C stretching modes of dipalmitoyl phosphatidylcholine was determined for the gel, phase transition and liquid crystalline states of the lipid multilayers. The van't Hoff enthalpy differences $\Delta H_{vH}$

between trans and gauche rotational isomers were obtained from the Raman spectral data for the temperature region characteristic of each bilayer state. An average size of about 30 molecules for the cooperative unit undergoing the chain melting process during the phase transition was estimated from the ratio of the appropriate van't Hoff enthalpy to a corrected calorimetric enthalpy. (Levin and Yellin)

(3) Although normally a difficult determination, infrared spectra were obtained as a function of temperature for a variety of phospholipid/water bilayer assemblies (80% water by weight) in the 3000-950 $cm^{-1}$ region. Spectral band-maximum frequency parameters were defined for the 2900 $cm^{-1}$ hydrocarbon chain methylene symmetric and asymmetric stretching vibrations. Temperature shifts for these band-maximum frequencies provided convenient probes for monitoring the phase transition behavior of both multilamellar liposomes and small diameter single-shell vesicles of dipalmitoyl phosphatidylcholine/water dispersions. As examples of the effects of bilayer components upon phase transition characteristics, the temperature profiles for lipid/cholesterol/water (3:1 mol ratio) and lipid/cholesterol/amphotericin B/water (3:1:0.1 mol ratios) vesicles were examined using the methylene stretching frequency indices. In comparison to the pure vesicle form, the transition width of the lipid/cholesterol system increased by nearly a factor of two (to 8°C) while the phase transition temperature remained approximately the same (41°C). For the lipid/cholesterol/amphotericin B system, the phase transition temperature increased by about 4.5°C (to 45.5°C) with the transition width increasing by nearly a factor of four (to ~15°C) above that of the pure vesicles. The lipid/cholesterol/amphotericin B data were interpreted as reflecting the formation below 38°C of a cholesterol/amphotericin B complex whose dissociation at higher temperatures (38-60°C range) significantly broadens the gel-liquid crystalline phase transition. (Levin and Asher)

(4) As a prelude to using deuterated membrane lipids as further probes of the behavior of the individual monolayers forming the discrete bilayer unit, vibrational Raman spectra of polycrystalline 1,2-dipalmitoyl phosphatidylcholine-$d_9$ (fully deuterated choline methyl groups) and 1,2-dipalmitoyl phosphatidylcholine-$d_{62}$ (fully deuterated acyl chains) were recorded and analyzed in the 3050-2800, 2250-2050 and 1800-700 $cm^{-1}$ regions. The fundamental vibrational modes were assigned primarily on the basis of isotopic frequency shift ratios, group frequency correlations and comparisons with specific model compounds. Since deuterium substituted lipids provide well-isolated spectral probes, particularly in the carbon-deuterium stretching region, the dependence of the 2250-2050 $cm^{-1}$ region on lipid phase was examined for the dipalmitoyl phosphatidylcholine-$d_{62}$ species. The methylene $CD_2$ deformation and twisting modes at 984 and 919 $cm^{-1}$, respectively, also exhibit intense, isolated vibrational transitions which should prove useful for monitoring molecular order in both mixed deuterated and undeuterated lipid systems. Except for the relatively weak choline methyl C-D and C-H stretching modes, the spectrum of 1,2-dipalmitoyl phosphatidylcholine-$d_9$ is not distinguishable from that of the undeuterated system.

For both the $-d_9$ and undeuterated species, the vibrational modes associated with the lipid head group region are sensitive to slight hydration. (Levin and Bunow)

(5) The 3000-2800 $cm^{-1}$ C-H stretching region of both phospholipids and normal hydrocarbon systems still presents difficulties in correctly interpreting the intensity distributions of the Raman spectroscopic contours. In particular the broad, environmentally sensitive 2935-2910 $cm^{-1}$ feature in the vibrational Raman spectra of phospholipids originates primarily from methylene asymmetric carbon hydrogen (C-H) stretching modes. For ordered acyl chains exhibiting local $C_{2h}$ symmetry at low temperatures, the infrared active methylene C-H asymmetric stretching mode becomes increasingly apparent at higher temperatures in the Raman spectrum as intramolecular chain disorder (trans-gauche isomerization) leads to a loss of chain symmetry. This effect dramatically changes the appearance of the Raman contour as the system specifically undergoes a gel-liquid crystalline phase transition. The 2935 $cm^{-1}$ shoulder, assigned to the acyl chain methyl group C-H symmetric stretching vibration, is difficult to distinguish as a separate spectral transition in the chain disordered state. For a variety of phospholipids an ordering of lipid fluidity was discussed in terms of the intensities of features in the 3000-2800 $cm^{-1}$ C-H stretching region. Particular emphasis was placed upon the 2935-2910 $cm^{-1}$ portion of the contour. Since the C-H stretching region for the vibrational Raman spectra of biological membranes often provides the most intense spectral transitions, an understanding of these detailed vibrational assignments becomes critical in interpreting structural reorganizations with bilayer systems. (Levin and Bunow)

(6) Polymorphic crystals of dipalmitoyl lecithin and lysolecithin, extracted from a variety of solvents, were examined by both infrared and Raman spectroscopic techniques in order to assess the subtle conformational changes accessible to lipid systems. We have suggested vibrational assignments which enable one to monitor increasing disorder in both the hydrocarbon chain and polar head regions. (Hill and Levin)

Significance to Biomedical Research and the Program of the Institute:

Raman spectroscopy offers a potentially valuable approach toward examining dynamical properties of natural and artificial membrane bilayer assemblies since no membrane perturbing agent is introduced by the technique. This is particularly crucial for investigating conformation changes induced in bilayer systems either by changes in temperature or by addition of membrane substituents.

Proposed Course of Project:

Investigations of the vibrational behavior of both vesicle and multilamellar phospholipid bilayer systems will continue with emphasis on (a) lipid-protein interactions by means of resonance Raman techniques using tunable dye laser procedures in the visible spectral region and selected

laser excitation frequencies in the ultraviolet spectral region, (b) compositional bilayer asymmetries induced by effects of surface curvature, (c) the use of deuterated lipid species to differentiate between "in-side" and "out-side" bilayer surfaces, and (d) the detailed manner in which cholesterol-phospholipid complexes form within membrane bilayers.

Publications:

Spiker, R.C., Jr., Pinnavaia, T.J. and Levin, I.W.: Use of phospholipid-clay complexes for determining vibrational spectra of membrane related systems. Biochim. Biophys. Acta 455: 588-596, 1976.

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Bunow, M.R. and Levin, I.W.: Location of Membrane Components Determined by Raman Spectroscopy. Proceedings of the Joint USA-USSR Symposium on Ion Transport Across Biological Membranes. New York, Raven Press. In press.

Yellin, N. and Levin, I.W.: Hydrocarbon chain trans-gauche isomerization in phospholipid bilayer gel assemblies. Biochemistry 16: 642-647, 1977.

Bunow, M.R. and Levin, I.W.: Comment on the carbon-hydrogen stretching region of vibrational Raman spectra of phospholipids. Biochim. Biophys. Acta 487: 388-394, 1977.

Yellin, N. and Levin, I.W.: Cooperative unit size in the gel-liquid crystalline phase transition of dipalmitoyl phosphatidylcholine-water multilayers: an estimate from Raman spectroscopy. Biochim. Biophys. Acta. In press.

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Abramowitz, S., Acquista, N. and Levin, I.W.: Infrared spectra of matrix isolated $FeO_2$: evidence for a cyclic iron-oxygen complex. Chem. Phys. Lett. In press.

Bunow, M.R. and Levin, I.W.: Raman spectra and vibrational assignments for deuterated membrane lipids: 1,2-dipalmitoyl phosphatidylcholine-$d_9$ and -$d_{62}$. Biochim. Biophys. Acta. In press.

Yellin, N. and Levin, I.W.: Hydrocarbon chain disorder in lipid bilayers: temperature dependent Raman spectra of 1,2-diacyl phosphatidylcholine-water gels. Biochim. Biophys. Acta. In press.

REPORTED IN PRESS 1976

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| SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space) | U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT | PROJECT NUMBER Z01-AM-29002-04-LCP |
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PERIOD COVERED
July 1, 1976 through September 30, 1977

TITLE OF PROJECT (80 characters or less)

Chemistry and biosynthesis of natural compounds, and instrumental methods used in their study

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

| | | | |
|---|---|---|---|
| PI: | Ulrich Weiss | Research Chemist | A LCP |
| OTHER: | Surendra P. Bhatnagar | Visiting Scientist | A LCP |

COOPERATING UNITS (if any)
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J. Michael Edwards, School of Pharmacy, University of Connecticut, Storrs
Iain C. Paul, Department of Chemistry, University of Illinois, Urbana

LAB/BRANCH
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SECTION
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NIAMDD, NIH, Bethesda, Maryland 20014

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SUMMARY OF WORK (200 words or less - underline keywords)

2-Fluoromorphine has been prepared for study of its analgesic action and receptor-binding ability, in comparison with those of the 1-fluoro isomer. Synthesis of codeine and/or 1-fluorocodeine from precursors suitably blocked to prevent formation of undesired products is being investigated. The structure of a long-known anomalous demethylation product of 5-benzyldihydromorphinone has been elucidated. The color dimorphism of 14-hydroxymorphinone has been studied; it is connected with different intermolecular hydrogen-bonding in the colorless and yellow forms, respectively. Tetramethyl cis-bicyclo[3.3.0]octane-3,7-dione 2,4,6,8-tetracarboxylate loses the carbomethoxyls at C-4 and C-8 almost quantitatively on mild treatment with NaOMe in DMSO-MeOH. Tricyclo[6.3.0.$0^{1,5}$]undecane-3,7,9-trione, derived from a little-known, conformationally rigid ring system, has been synthesized. These cyclopentanoid compounds might be valuable intermediates in the synthesis of pharmaca. Reaction of dimethyl 3-ketoglutarate with glyoxal and guanidine did not yield the expected derivative of cyclopentano-4,5-imidazolidine. Lachnanthocarpone, a 9-phenylphenalenone pigment from Lachnanthes tinctoria (Haemodoraceae) has been synthesized by spontaneous intramolecular Diels-Alder reaction of a suitable orthoquinonoid 1,7-diarylheptadiene.

Project No.: Z01-AM-29002-04-LCP

Project Description:

Objectives:

Isolation in pure form, and elucidation of the chemical constitution of a variety of naturally occurring substances of chemical, biosynthetic, or biological interest. Study of instrumental techniques used in such work. Corroboration of chemical structure by total synthesis. Synthesis of compounds of medical or biological interest.

Methods Employed:

Standard procedures of organic chemistry, and instrumental methods (UV, IR, NMR spectroscopy). For measurement of optical rotatory dispersion and circular dichroism, the Cary 60 spectropolarimeter and its CD attachment are used.

Major Findings and Future Plans:

1. Analgesics of the Morphine Series

a) 2-Halogenocodides and -morphides. We have observed earlier (see report for 1973/74 and 1974/75) that 1-fluorocodeine is very similar to codeine itself with respect to analgesic activity in animals and to binding to the narcotics receptor of the central nervous system, while the analogous 1-chloro and 1-bromo compounds have much diminished action. This decrease in activity is thus caused by the bulk of the substituents rather than by their electronegativity.

This work has now been extended to the 2-halogenomorphines and -codeines. Their preparation is being studied by Dr. N. Chatterjie under contract with NIAMDD, and by Dr. Kenneth L. Kirk, LC-NIAMDD. So far, 2-fluoromorphine has been obtained, but remains to be more fully characterized; work on the preparation of 2-bromomorphine is under way. It is planned to prepare the entire series of 1- and 2-fluoro-, chloro-, bromo-, and iodomorphines and -codeines for a comparative study of their analgesic activity and receptor-binding affinity. The results of such a study should yield additional information on the nature of the interaction of analgesic drugs with the specific receptor. Furthermore, the 2-fluoro compounds might be of pharmacological or therapeutic interest, since the fluorine atom should be hydrogen-bonded to the phenolic hydroxyl next to it, and its powerful electronegativity should make this hydroxyl more acidic. Both effects may be expected to increase the intensity and/or duration of the analgesic action.

b) Biomimetic Synthesis of Morphine and Codeine or of their 1-Fluoro Derivatives. (S. P. Bhatnagar) See report for 1975/76. The sequence of steps by which the opium alkaloids thebaine, codeine, and morphine are produced in the opium poppy from their precursor, the 1-benzyl-tetrahydroisoquinoline alkaloid reticuline, is well understood, and all reactions

involved have been duplicated in the laboratory. Use of this sequence for a practical synthesis would thus appear very promising, but realization of this goal has so far been prevented by the fact that one key step proceeds very poorly in the laboratory: in the intramolecular phenol-coupling leading from reticuline to the next intermediate, salutaridine, the desired coupling mode is a relatively minor reaction (at best ~23% yield achieved through the use of a highly undesirable thallium-containing reagent), while formation of two other alkaloids, isoboldine and isosalutaridine (pallidine) is favored; these two bases can not be converted to codeine or morphine. Their formation involves position 6' of reticuline, while that of salutaridine takes place through position 2'. Consequently, we decided to explore the phenol-coupling of derivatives of reticuline in which position 6' is blocked by a suitable substituent. It is known that carbon 6' of reticuline becomes carbon 1 of codeine and morphine.

Several choices of blocking substituents are available. A priori, a substituent would be desirable which would be removed later on. Bromine is the obvious choice, and is widely used in similar situations; in the present case, it would lead to the known 1-bromocodeine which is easily reduced to codeine by lithium aluminum hydride. However, in two earlier attempts reported in the literature some time ago, the bromine was lost from the molecule during the coupling reaction, isoboldine being formed. Modern improved methods might be capable of avoiding this difficulty, and should obviously be explored, but it seemed preferable to investigate other, more tightly bound substituents first, in order to establish whether the reaction still takes place in their presence. We decided to study the use of the nitro and methyl-thio groups, and of fluorine. The two former substituents can be removed by known methods after they have fulfilled their purpose. In contrast, the fluorine atom is so tightly bound that no general technique is known for its elimination from an aromatic ring; the synthesis in this case could only yield 1-fluorocodeine, but not codeine itself. Since the fluoro compound is known to be as active as codeine itself (at least in animals), this fact is not too objectionable, although the well-known difficulties in getting acceptance for a new drug constitute a serious drawback.

Preparation of the intermediates necessary for the synthesis of 6'-nitro- and -fluororeticuline has been studied in detail. The nitro compound is particularly desirable, since the nitro group can be exchanged for F and SMe by known methods. Initial difficulties were caused by the unexpected ease with which this group participated in some of the reactions; two new compounds of so far unknown structure have been obtained as a consequence. However, key intermediates for the preparation of both nitro- and fluororeticuline are now in hand. The introduction of fluorine has been carried out by Dr. Kirk with the use of his photochemical method.

This research is to be completed in cooperation with Dr. Bhatnagar after his return to India.

c) Identification of an Alleged Isomer of 5-Benzyldihydrocodeinone. (S. P. Bhatnagar) During his extended studies on transformation products of morphine and codeine, the late Dr. L. F. Small, then Chief, LC-NIAMDD, had prepared a series of alkylated dihydrocodeinones, which could be demethylated with HBr in the usual manner to the corresponding alkyl-dihydromorphinones; the alkyl groups were found later to occupy position 5. The demethylations proceeded normally in all cases except that of the benzyl derivative, where the expected, alkali-soluble 5-benzyldihydromorphinone was accompanied by another compound. On the basis of its alkali-insolubility and of an elementary analysis, Small assumed that demethylation had failed to take place during the formation of this compound, which seemed to be isomeric with 5-benzyldihydrocodeinone, and to be formed from it through some rearrangement. No further evidence on the structure of this substance was obtained. Since the product of such a rearrangement could be chemically and pharmacologically interesting, a sample remaining from Dr. Small's work, made available by Dr. E. L. May, was investigated sporadically during the last ∿10 years by modern instrumental methods. Contrary to the original interpretation, the expected demethylation was found to have taken place during the formation of the compound, which is indeed phenolic (bathochromic shift of the UV absorption on addition of NaOH to the alcoholic solution). High-resolution mass spectrometry showed that the substance was formed through loss of the methyl group and of one molecule of water. The empirical formula so derived is actually in better agreement with Small's elementary analysis than the one favored by him. We found that the compound is a product of further transformation of 5-benzyldihydromorphinone, and can be prepared from it by prolonged heating with HBr. Almost all available information on the substance is compatible with a structure which permits interpretation of its formation in a way which has close precedent in certain reactions of compounds unrelated to codeine. This structure is formed by interaction of the carbonyl of 5-benzyldihydromorphine with the aromatic ring of the benzyl group, followed by elimination of water, so that a styrene chromophore is formed. Only the insolubility in alkali remains unexplained; it may be due to formation of an insoluble Na salt, but existence of this doubtful point makes confirmation of our structure by X-ray crystallography desirable. Since the supply of material is now exhausted, the somewhat laborious preparation of the compound will have to be repeated in Dr. Bhatnagar's laboratory in India.

d) Color Dimorphism of 14-Hydroxymorphinone. This phenolic compound, first described by me in 1957, shows the peculiarity of crystallizing in bright yellow square leaflets from ethanol or acetone, in perfectly colorless needles from benzene. No similar dimorphism is observed in such closely related compounds as 14-hydroxycodeinone (the non-phenolic methyl ether), oxymorphone (the dihydro derivative), or morphinone (identical except for lack of the hydroxyl in position 14). Differences in the frequency of the IR band due to the α,β-unsaturated carbonyl group present in 14-hydroxymorphinone suggested that this group is hydrogen-bonded in the yellow, but not in the colorless form; consequently, the dimorphism might be due to different ways in which the molecules are held together by hydrogen bonds in

the crystal lattices of the two forms. Interpretation of the two modifications as keto and enol form is ruled out: 14-hydroxymorphinone is non-enolizable.

Recent X-ray crystallographic work by Prof. Iain C. Paul (University of Illinois, Urbana) has shown that this hypothesis is indeed correct, and that, as anticipated, the carbonyl is involved in hydrogen bonding in the yellow form only. The cause of the striking difference in visible color is not yet clear. It seems possible that the color of the yellow form is due to the fact that its carbonyl is held above the phenolic ring of another molecule in such a way that charge-transfer interaction should be possible. This problem is being studied further.

2. Reaction of Dimethyl β-Ketoglutarate with Dicarbonyl Compounds

The structure of the hexahydrobenz[e]indene mentioned in Section 3a of last year's report had been derived mostly on the basis of nmr spectroscopy. Because of the interest of the compound as a potential intermediate for the total synthesis of steroids, an X-ray crystallographic study was undertaken at our request by Drs. J. V. Silverton and G. J. Shaw (IR CH, NHLBI). Their work has completely confirmed the proposed structure and has clarified one point of stereochemistry on which no evidence had been available.

The further steps required to convert the compound into various steroids are being studied by Prof. Habermehl and his students at the Technische Hochschule, Darmstadt, Germany.

During attempts to utilize tetramethyl cis-bicyclo[3.3.0]octane-3,7-dione 2,4,6,8-tetracarboxylate (very easily made by reaction of dimethyl β-ketoglutarate with glyoxal in aqueous solution at room temperature) for the preparation of substances of potential biomedical value, Dr. Bhatnagar discovered an unexpected, smooth reaction of the compound with sodium methoxide in a mixture of dimethyl sulfoxide and methanol; in this process, the carbomethoxyls in positions 4 and 8 are removed, and dimethyl cis-bicyclo-[3.3.0] octane-3,7-dione 2,6-dicarboxylate is produced in almost quantitative yield. The product of removal of only one carbomethoxy group seems to be an intermediate; it can be isolated, in very low yield, if the reaction is run under milder conditions. The dicarbomethoxy compound, in turn, loses one of its carbomethoxyls only with great difficulty under strenuous conditions; a 1% yield of the monocarbomethoxy-bicyclooctanedione was obtained, the rest of the material being unchanged.

The continued cooperation with Prof. J. M. Cook and his coworkers has yielded another cyclopentanoid, tricyclo[6.3.0.$0^{1,5}$]undecane-3,7,9-trione. This compound can be made easily by reaction of 4,5-dioxovaleric acid readily accessible from technical levulinic acid) with dimethyl β-ketoglutarate, acid-catalyzed decarboxylation of an intermediate, and cyclization of the resulting compound under acidic conditions. The tricyclic triketone

so prepared is derived from a ring system made up of three five-membered rings. Derivatives of this ring system had been obtained only once before, quite recently, by involved transformations, while our method provides a very convenient approach. Models of the ring-system show complete absence of conformational mobility; since such conformationally rigid compounds have been found repeatedly to have valuable biological actions, our trione has definite potentialities as starting material for drugs.

The starting point for our investigation of the reaction of dimethyl β-ketoglutarate with dicarbonyl compounds had been an attempt in 1967, with Dr. J. M. Edwards, to work out a synthesis of the ring system of biotin sulfone through reaction of dimethyl sulfonyldiacetic acid, glyoxal, and and urea or a related compound. In an orienting first experiment, the commercially available dimethyl β-ketoglutarate was substituted for the analogous, less readily accessible sulfur compound. However, the products isolated from this reaction (seven substances so far) were invariably derived from dimethyl β-ketoglutarate and glyoxal; no compound formed with participation of the urea has ever been encountered. This failure to enter the reaction might conceivably be due to the fact that urea is an essentially neutral compound. During his one-month's period of work at NIH last summer, Dr. Edwards therefore repeated the initial attempt, modifying it by the use of the strongly basic guanidine in place of urea. Again, only products resulting from reaction of the two nitrogen-free reactants were obtained under a variety of conditions. Furthermore, 4,5-dihydroxy-imidazolidin-2-one, the product of the reaction of urea with glyoxal, failed to react with dimethyl β-ketoglutarate, but parabanic acid (imidazolidine-2,4,5-trione) reacted with the ketoglutarate in alkaline medium to give a good yield of a compound which remains to be identified.

The cooperation with Profs. J. M. Cook and G. Habermehl is to be continued.

3. 9-Phenylphenalenone Pigments from Plants of the Family Haemodoraceae

Pigments derived from 9-phenylphenalenone have been found in every species of the small monocotyledonous family Haemodoraceae which has been available for study so far; they have never been found in any other organism. Research on the occurrence, chemistry, biosynthesis, and biological activity of these pigments has been carried out in cooperation with Dr. J. M. Edwards since 1967. The biosynthesis of the 9-phenylphenalenones in particular presents a very unusual problem, and is still incompletely understood. It was postulated years ago by R. Thomas that they form through cyclization of compounds derived from 1,7-diarylheptane, a class of natural compound typified by curcumin, the long-known main pigment of turmeric; however, no assumption on the actual mode of this cyclization was made.

It seemed possible that the ring system of the 9-phenylphenalenones could be formed through an intramolecular Diels-Alder reaction of a diarylheptanoid orthoquinone with a pair of conjugated double bonds in the unbranched seven-carbon chain.

A. C. Bazan in Dr. Edwards' laboratory has now prepared such an ortho-quinone by the oxidation of 1-phenyl-7-(3',4'-dihydroxyphenyl)hepta-1,3-diene-5-one with aq. $NaIO_4$. The dihydroxy compound itself was synthesized following a route worked out by a Japanese group for the synthesis of the analogous diene lacking the two hydroxyls; this diphenylheptadienone is a constituent of a plant quite unrelated to the Haemodoraceae. The ortho-quinone was found to cyclize spontaneously, giving a surprisingly good yield of lachnanthocarpone, one of the phenylphenalenones isolated earlier by us from Lachnanthes tinctoria, the only native haemodoraceous plant. This formation of the ring system of the natural pigment can hardly proceed otherwise than by the postulated intramolecular Diels-Alder reaction; however, the primary product of this reaction, which was not observed, would have to undergo dehydrogenation to give the phenalenone system. In any event, this synthesis proves that such a reaction is actually feasible, and thereby strengthens the hypothesis of Thomas, although it evidently does not prove it. No Diels-Alder reaction has ever been demonstrated experimentally to play a role in biosynthesis, although the structures of a few naturally occurring compounds definitely suggest that such reactions occur during their formation.

Publications:

Yang-Lan, S., Mueller-Johnson, M., Oehldrich, J., Wichman, D., Cook, J.M. and Weiss, U.: Reaction of dicarbonyl compounds with dimethyl β-ketoglutarate. 4. Formation of 1:1 adducts. J. Org. Chem. 41: 4053-4058, 1976.

Oehldrich, J., Cook, J.M. and Weiss. U. Reaction of dicarbonyl compounds with dimethyl β-ketoglutarate. V. Simple synthesis of tricyclo[6.3.0.$0^{1,5}$]undecane-3,7,9-trione, a novel cyclopentanoid compound. Tetrahedron Letters: 4549-4522, 1976.

Bazan, A.C., Edwards, J.M. and Weiss, U.: Synthesis of lachnanthocarpone [9-phenyl-2,6-dihydroxyphenalen-1(6)-one] by intramolecular Diels-Alder reaction of a 1,7-diarylheptadienoid intermediate. Tetrahedron Letters: 147-150, 1977.

Rice, K.C., Weiss, U., Silverton, J.V. and Shaw, G.J.: Reaction of dimethyl 3-ketoglutarate with 1,2-dicarbonyl compounds. V. Simple synthesis of derivatives of 2,3,3a,4,5,9b-hexahydro-1H-benz[e]indene from dimethyl 3-ketoglutarate and glyoxal. J. Org. Chem. In press.

Bhatnagar, S.P., Weiss, U. and Highet, R.J.: Reaction of dimethyl 3-ketoglutarate with 1,2-dicarbonyl compounds. VIII. Selective base-catalyzed decarbomethoxylation of tetramethyl cis-bicyclo[3.3.0]octane-3.7-dione 2,4,6,8-tetracarboxylate; preparation of 2,6-dicarbomethoxy-cis-bicyclo[3.3.0]octane-3,7-dione. J. Org. Chem. In press.

| SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space) | U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT** | PROJECT NUMBER Z01-AM-29003-10-LCP |
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PERIOD COVERED
July 1, 1976 to September 30, 1977

TITLE OF PROJECT (80 characters or less)

The physical chemistry of membranes and complex membrane systems of biological interest.

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: Gerald M. Shean Research Chemist A LCP

COOPERATING UNITS (if any)
Karl Sollner (Scientist Emeritus) A:LCP

LAB/BRANCH
Laboratory of Chemical Physics

SECTION
Molecular Biophysics

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20014

| TOTAL MANYEARS: | PROFESSIONAL: | OTHER: |
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| 1 | 1 | 0 |

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

It was demonstrated that the same mosaic membrane may be selectively anion permeable for transport processes and selectively cation permeable for electrical processes. This was accomplished by using a mosaic membrane consisting of a high resistance (275,000Ω) liquid anion exchanging membrane in parallel with a fairly high resistance (9,580Ω) porous cation exchanging membrane. When this mosaic membrane seaparted .05 N RbSCN from .1 N RbSCN, a concentration potential resu 1 with the more dilute side positive. This indicated that the membrane was more electrically permeable to cations than anions. The self-exchange transport rates as determined with radioactive isotopes showed, however, that the thiocyanate anion was transported over 400 times faster than the rubidium cation across the membrane. The main reason for this apparent conflict is that the anions were transported across the membrane in an essentially undissociated form. Their rate was not dependent upon the electrical potential of the mosaic membrane nor upon the electrical resistance of the liquid anion permeable membrane part. The demonstration is of value in understanding that similar processes may be workin5 in the much more complex biological membrane systems. It cautions against

routinely ascribing to a biological membrane only one type of ion selectivity for both electrical potential and transport properties.

Project Description:

Objectives:

To prepare and study well-defined synthetic porous and liquid ion-exchanger membranes and membrane systems with the ultimate aim to provide a rational basis for the understanding of various complex membrane phenomena occurring in living systems.

Methods Employed:

The methods employed were essentially the same as reported previously, including flame photometry, potentiometric titrations (including the chloridimeter), spectromety, non-aqueous titrations, etc.

Major Findings:

A new type of mosaic membrane system was investigated. It consists of a porous cation-selective membrane placed in parallel with a liquid anion-selective membrane. Electrical potentials and transport rates of ions across the system were measured. The objective was to demonstrate that for a mosaic membrane system, the ionic selectivity as determined by electrical potential measurements does not necessarily indicate the ion transport selectivity and vice versa. This objective was reached.

For instance, in a concentration cell mosaic membrane system:

.05 N RbSCN | mosaic membrane | .1 N RbSCN

a potential of 10.2 mV was found. The more dilute side was positive in sign, indicating that the system was cation selective (i.e. allowing a much greater rate of cations to cross than anions). However, an analysis of the rates of transport across the system of the $Rb^+$ and the $SCN^-$ showed that the thiocyanate anions were transported over 400 times more rapidly than the rubidium cations.

The cause of this apparent conflict of results rests with the fact that transport rates through the liquid membrane part of the mosaic system are only peripherally involved with the electrical potential or electrical resistance of the system (or of the liquid membrane part of the system). By having a liquid anion exchanger membrane that was over 25 times the resistance of the porous cation exchanger membrane in which it was electrically in parallel, the mosaic system was for potential purposes dependent on the porous membrane and therefore was cation selective. The porous cation exchanger membrane's resistance (9,580 ohms), however, was so high that the exchange rate of cations was very low. By contrast, the anion exchange rate was quite high due to stirring of the liquid membrane, which essentially diminished its thickness to that of the Nernst layers at the membrane interfaces. This transport of anions

in an undissociated state (i.e. associated with the ion-cxchanger) is not indicated electrically.

Significance to Biomedical Research and the Program of the Institute:

Understanding of the complex membrane phenomena in living cells and tissues is helped by information gained from simple membrane model systems that can reproduce some of the phenomena that occur in living systems. Model studies indicate the possibility of easy explanations for phenomena that have previously been thought of as very complicated. The demonstration reported here has implications in biological membrane studies where it is often assumed that there is only one type of selectivity across a membrane. The danger in this assumption is shown by this mosaic membrane system that electrically behaves as though it were a cation permeable membrane, while it behaves transport-wise as an anion permeable membrane.

Proposed Course of Project:

The use of mosaic membranes employing liquid ion-exchange membranes in parallel with porous ion-exchange membranes will be expanded by using different electrolyte concentration ratios and different electrolytes in the same cell. Models that will demonstrate accumulations of electrolytes from dilute solutions will also be investigated.

Publications:

Shean, G. M.: Bi-ionic potentials across liquid cation exchanger membranes, J. Membr. Sci., in press.

| SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space) | U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT** | PROJECT NUMBER Z01-AM-29004-08-LCP |
|---|---|---|

PERIOD COVERED
July 1, 1976 through September 30, 1977

TITLE OF PROJECT (80 characters or less)

Photochemical reactions and their mechanisms

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

| | | | |
|---|---|---|---|
| PI: | Herman Ziffer | Research Chemist | A LCP |
| OTHER: | Mitsuru Imuta | Visiting Fellow | A LCP |

COOPERATING UNITS (if any)

LAB/BRANCH
Laboratory of Chemical Physics

SECTION
Section on Molecular Biophysics

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20014

| TOTAL MANYEARS: | PROFESSIONAL: | OTHER: |
|---|---|---|
| 0.5 | 0.5 | |

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

As a result of a study of the effect the reaction temperature has on the distribution of major (cis A/B ring junction)/minor (trans A/B ring junction) photoproducts of A-homocholest-4a(5)-en-3-one, we found that the yield of the minor product could be increased by increasing the temperature of the irradiation solution.



PHS-6040
(Rev. 10-76)

Project No.: Z01-AM-29004-08-LCP

Project Description:

In order to permit a more rational use of photochemical reactions in synthesis, a better understanding of the mechanism and stereochemical requirements of these reactions is necessary. In an earlier study of the photochemical rearrangement of A-homocholest-4a(5)-en-3-one we showed that cis and trans isomeric photoproducts were formed with the cis-compounds predominating. If the irradiation is carried out in xylene, at higher temperatures, the ratio of trans/cis isomers increased. We wish to use the above information in directing the course of the photochemically induced rearrangement of a D-bishomo β,γ-unsaturated ketone. The major product is expected to contain an α-vinyl group at C-13. While such steroids with an unnatural configuration at C-13 have rarely been tested for androgenic activity, the more promising compound is the β-vinyl derivative. It is the yield of the latter compound we hope to improve by conducting the irradiation in xylene at higher temperature. Synthesis of the starting material for the irradiation has begun.

| SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space) | U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT | PROJECT NUMBER Z01-AM-29005-03-LCP |
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PERIOD COVERED
July 1, 1976 through September 30, 1977

TITLE OF PROJECT (80 characters or less)
Asymmetric synthesis; structure, stereochemistry and NMR

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

| | | | |
|---|---|---|---|
| PI: | Herman Ziffer | Research Chemist | A LCP |
| OTHER: | Mitsuru Imuta | Visiting Fellow | A LCP |

COOPERATING UNITS (if any)

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NIAMDD, NIH, Bethesda, Maryland 20014

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

A series of benz-α-tetralone derivatives have been prepared and reduced using Sporobolomycetes pararoseus. Each ketone yielded an optically active alcohol whose absolute stereochemistry was determined as [S] by chemically transforming the corresponding acetates to (-)-dimethyl α-acetoxyadipate and (-)-dimethyl α-acetoxyglutarate. In addition, several other groups of compounds were reduced, establishing that (1) the specificity of the reduction was not limited to cyclohexanone derivatives, and (2) the presence of substituents α- to the carbonyl did not alter the course of the reduction. We have also prepared optically active amines from these α-tetralol derivatives, as well as the enantiomeric alcohols. We have examined the reduction of a series of substituted benzil derivatives (α-diketones) which yields essentially optically pure threo diols. The diols have been converted to trans-stilbene oxides; the unsymmetric oxides were hydrolyzed to yield the optically active erythro-diols. This observation requires a regiospecific attack by water, a reaction that could not be predicted on the basis of available information. These compounds have been used to test predicted relationships between optical properties (signs of CD bands) and absolute stereochemistry.

Project No.: Z01-AM-29005-03-LCP

Project Description:

For a variety of studies in pharmacology and molecular biology it is very desirable and frequently essential to have both enantiomers of the compound under investigation in order to distinguish between specific and non-specific effects. We have therefore extended our original studies on asymmetric syntheses to develop procedures for preparing compounds of high optical purity and for converting the alcohols obtained to amines and other derivatives. In our earlier studies, we found that microbiological reductions of hydroaromatic ketones yielded alcohols of a predictable absolute stereochemistry and in high optical purity. In asymmetric chemical reductions optical yields vary widely and the absolute stereochemistry of the products is unpredictable. In order to improve the yields in the microbiological reductions, we screened a variety of micro-organisms and now have one strain with the same "product stereospecificity" as Sporobolomycetes pararoseus but yielding 3 to 5 times more alcohol.

We have continued studies on the reduction of phenyl ketones and shown that: (1) The stereospecificity of the reduction is not limited to cyclohexanone derivatives, i.e. acyclic, five and seven membered cycloalkanones all yield (S) alcohols. (2) The presence of a substituent α- to the carbonyl does not affect the configuration of the alcohol formed. Thus, when 2-acetoxy-α-tetralone is used as a substrate, a mixture of cis and trans diol monoacetates is formed. These diols are dihydro derivatives of compounds derived from metabolism of naphthalene by animals or plants.

In order to demonstrate the value of these microbiological reductions we have developed reaction sequences that convert an optically active alcohol into its enantiomer, or replace the hydroxyl group with an amino group without concomitant racemization.

We have also examined the reduction of a series of α-diketones (benzil derivatives) which yield essentially optically pure substituted threo 1,2-diphenylethane diols. Threo diols in this series are frequently troublesome to prepare and it is often difficult to determine whether a diol has the threo or erythro configuration. The most reliable method for assigning stereochemistry of symmetric 1,2-diols involves resolving the threo compound. Since these reductions yield optically active threo diols (symmetric erythro diols are meso) the method is valuable for the synthesis of threo diols and as a means of distinguishing threo from erythro-compounds. The absolute stereochemistry about the hydroxyl-bearing carbons in these compounds is the same as that of the hydroaromatic alcohols.

The threo-diols synthesized by these microbiological reductions have been used in several studies. In one of these, the diols were converted to cis stilbene oxides, and the relation between the sign of the short wavelength CD bands and their absolute stereochemistry was investigated. In another study the unsymmetric threo-diols were converted to the cis-stilbene oxides and the hydrolysis of these compounds was examined. The products were

the optically active erythro diols, which requires that the attack by water on the oxide is regiospecific. The regiospecificity is unexpected and is currently being investigated.

Publications:

Dansette, P.M., Ziffer, H. and Jerina, D.M.: Optically active 4-substituted cis-1,2-diphenylethylene oxides and related 1,2-diphenylethane diols. Tetrahedron 32: 2071-2074, 1976.

Kabuto, K., Shindo, H. and Ziffer, H.: Synthesis, stereochemistry, $^{13}C$ NMR, and chiroptical properties of isomeric 1,2-dihydroxy-3-phenylcyclohexanes. J. Org. Chem. 42: 1742-1746, 1977.

Ziffer, H., Kabuto, K., Gibson, D.T., Kobal, V.M. and Jerina, D.M.: The absolute stereochemistry of several cis-dihydrodiols microbially produced from substituted benzenes. Tetrahedron. In press.

| SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space) | U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT** | PROJECT NUMBER Z01-AM-29006-07-LCP |
|---|---|---|

PERIOD COVERED

July 1, 1976 to September 30, 1977

TITLE OF PROJECT (80 characters or less)

The relationship between the nuclear and electronic configuration of molecules and their spectroscopic and dynamic properties.

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

| | | | |
|---|---|---|---|
| PI: | Elliot Charney | Research Chemist | A LCP |
| OTHER: | Joan Rosenfield | Staff Fellow | A LCP |
| | Holly Ho Chen | Guest Worker | A LCP |

COOPERATING UNITS (if any)

Kiwamu Yamaoka, University of Hiroshima, Japan

LAB/BRANCH

Laboratory of Chemical Physics

SECTION

Spectroscopy and Structure

INSTITUTE AND LOCATION

NIAMDD, NIH, Bethesda, Maryland 20014

| TOTAL MANYEARS: | PROFESSIONAL: | OTHER: |
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| 4.5 | 3 | 1.5 |

SUMMARY OF WORK (200 words or less - underline keywords)

A. Molecular chiral phenomena are being examined by quantum mechanical calculations, (CNDO/S and CNDO/2), in organic compounds containing intrinsically dissymmetric conjugated systems.

B. Macromolecular structure and dynamics of large biological polymers, in particular polynucleotides, are being studied by electric-field induced dichroism and birefringence methods.



PHS-6040

Project No: Z01-AM-209006-07-LCP

Project Description:

This report covers projects in two different but allied fields: (1) Molecular chiral phenomena and (2) macromolecular structure and dynamics. The object of the first project is to try to increase our understanding of the nature of molecular chiral phenomena in general and in particular to see the extent to which quantum mechanical interpretation of molecular states provide a detailed explanation of the chirality of small molecules or individual chromophores. The objectives of the investigations into macromolecular structure and dynamics are to determine the molecular structure of polymers, biological polymers in particular, in determining their physical properties - and the converse as well. The special experimental methods employed have been described in previous reports and the theoretical methods are standard in the field.

1. Molecular Chiral Phenomena

a) The theoretical examination of the effect of allylic substituents on the chiral properties of the electronic transitions of dienes reported in progress last year has been extended to predict the sensitivity of the chiral activity to the rotomer distribution of the methyl group allylic to the diene. It has been found that the chiral activity is extremely sensitive to the particular rotamer chosen so that in the absence of free rotation of this group it cannot be treated as the smeared charge distribution of a carbon atom and its tetrahedral hydrogens. The theory has been applied to the circular dichroism of a conformationally mobile molecule, α-phellandrene, and is qualitatively in good accord with the observations. An examination of the rotamer distribution as a still more exacting test of theory is planned. Also underway is the calculation of the CD of two allylic groupings not previously examined as a further test of the theory to see whether the new treatment correctly predicts the observations on two new molecules reported to us by Andrew Galat of the Adam Mickiewicz University, Poznan, Poland.

b) The second observed transition in benzene has been assigned as a singlet $B_{1u}$ transition, part of the trio of degenerate states expected in the lowest energy π system of benzene. In a magnetic field, optical activity is induced in benzene, and the resulting MCD spectrum can be analyzed to determine whether the assignment based on the ordinary absorption is appropriate. The analysis of a highly structured MCD spectrum of a weak transition such as this band of benzene requires that the vibrational structure be interpretable. A theoretical treatment has been carried out in which the ordinary absorption and MCD vibronic spectrum of the $B_{1u}$ state of benzene have been predicted. The ordinary absorption intensities are in accord with the observed spectrum. The detailed vibronic predictions of the MCD spectrum await confirmation by a high resolution MCD experiment but the overall sign of the MCD band is in accord with a low resolution MCD spectrum.

2. Macromolecular Structure and Dynamics

The orientation of large polymeric molecules in pulsed electric fields and their disorientation when the fluid is turned off can be studied using monochromatic polarized light. These experiments are designed to obtain several types of information, including the molecular conformation or structure in solution (from the dichroism, which is the difference between the changes in extinction of the light polarized parallel and perpendicular to the electronic field when the molecules are completely oriented in the field) and the dynamics of intramolecular reorganization and of rotational disorientation (from the decay of the dichroism at the end of the field pulse).

a) We have continued and nearly completed the study of polyribose cytidilic acid poly(rC) reported last year and have made some new studies of polyribose adenylic acid. These latter measurements of the orientation of poly(rA) in different ionic strength solutions have been made to help elicit the polyelectrolyte behavior. The orientation has been found to be quite different from that expected from a classical uncharged polar polymer. The new experiments are designed to test a theory be Kikuchi and Yoshioka, [Biopolymers 15, 583 (1976)], for the field-induced orientation of a polyelectrolyte in dilute solution and simultaneously to determine whether the concept of the polynucleotide polyelectrolytes as rods with a uniform (rather than discrete) charge distribution adequately describes the behavior. Experiments to date tend to confirm this.

b) Another project, the results of which are as yet somewhat elusive, but which is concerned with an important experimental and theoretical problem, is a study of the equilibrium state obtained by mixing relatively monodispersed samples of two fractionated samples of poly(rC) under double stranding conditions. It is our contention, born out by the initial experiments but so far only with low precision, that the relatively monodispersed samples of both high and low molecular weight contain a significant amount of single stranded segments which tend to form more complete double strands in mixtures because of the increased availability of suitable size fragments.

Proposed Course of the Project:

The analysis of the field-induced orientation mechanism of the polynucleotide polyelectrolytes will be pursued with new experiments on the pH dependence of the orientation. We will also consider NMR experiments to determine the binding of counter ions to the polynucleotides under the conditions of the orientation experiments. Electric dichroism and birefringence experiments on polyriboseguanisine, poly(rG), are also underway. In these experiments we will be aided by the acquisition of a high speed Nicolet Explorer oscilloscope and interfaced Hewlett-Packard computer which will soon be added to our detection equipment.

Publications:

Rosenfield, J. S.: Calculation of vibronic magnetic rotational strengths and oscillator strengths in the $B_{1u}$ state of benzene. J. Chem. Phys. 66:921, 1977.

Project No: Z01-AM-209006-07-LCP

Rosenfield, J. S. and Charney, E.: Calculations of rotatory strengths in chiral chromophores: Cisoid conjugated dienes. J. Amer. Chem. Soc., 99:3209, 1977.

REPORTED IN PRESS - 1975

Charney, E.: Electro-optics in the infrared region. In O'Konski, C. T. (Ed.): Molecular Electro-Optics. New York, Marcel Dekker, 1976, pp. 381-399.

| SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space) | U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT** | PROJECT NUMBER Z01-AM-29007-06-LCP |
|---|---|---|

PERIOD COVERED

July 1, 1976 to September 30, 1977

TITLE OF PROJECT (80 characters or less)

Structure and interaction of paramagnetic biomolecules.

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

| | | | |
|---|---|---|---|
| PI: | Hideo Kon | Research Chemist | A LCP |
| OTHER: | Mitsuo Sato | Visiting Fellow | A LCP |
| | Amy S. Rispin | Guest Worker | A LCP |
| | Makoto Chikira | Visiting Fellow | A LCP |

COOPERATING UNITS (if any)

D. W. Nebert (CH-DPB)
W. M. Lovenberg (H-IR-HE)

LAB/BRANCH

Laboratory of Chemical Physics

SECTION

Spectroscopy and Structure

INSTITUTE AND LOCATION

NIAMDD, NIH, Bethesda, Maryland 20014

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| 3 | 3 | 0 |

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Continued effort has been made to investigate the model systems of the heme iron(III) in cytochrome P-450. On the experimental side, comparative EPR (electron paramagnetic resonance) studies were carried out for two different model systems and the various mammalian liver microsomal P-450. On the theoretical side, we have shown that the lowest excited $^4T_2$ state, which has not usually been included in the crystal field analysis of d-electron states of the high spin Fe(III) complexes, plays an important role in the systems with a substantial rhombic distortion. Other (collaborative) works are described.



PHS-6040
(Rev. 10-76)

Project No.: Z01-AM-29007-06-LCP

Project Description:

The purpose of this project is to clarify the structure-function relationship of the paramagnetic centers in various biological systems, especially the transition metal ions. Simple model systems have been used to simulate the actual biological molecules. Magnetic measurements are made by using EPR (electron paramagnetic resonance) spectroscopy and Faraday balance susceptometer, combined with cryogenic techniques. The ENDOR (electron nuclear double resonance) attachment to the EPR spectrometer is being fabricated. The data obtained are used to gain information on the electronic structure of the metal ion, the symmetry and the strength of the field surrounding the ion. The mode of interaction of the ion with the exogenous ligand can also be studied.

Major Findings:

1. (With Dr. Sato and Dr. Nebert) Continuation of #1 in 1975 report.

Comparative EPR studies have been made on cytochrome P-450 and two types of model systems--one being the high spin Fe(III) porphines with a thiolate axial ligand, and the other, high spin Fe(III) porphines diluted in corresponding free base porphines as mixed crystals. (No sulfur ligand is involved in the latter system.) As far as the g factors are concerned, these three systems have similar EPR characteristics showing large rhombic distortions. However, when comparison is made with regard to their dynamic properties such as temperature dependence of g shifts, and linewidth, or the microwave power saturation behavior, the distinction of the three systems becomes clear: the observed characteristics of cytochrome P-450 is qualitatively more similar to the sulfur containing model systems than with the mixed crystal system. An important exception is that the zero-field parameter D in cytochrome P-450 ($\sim$4 $cm^{-1}$) is much lower than in the model thiolates (8$\sim$12 $cm^{-1}$) and is close to D in the mixed crystal system (3$\sim$4 $cm^{-1}$). Thus, the P-450 heme has essential structural features in common with the thiolates but the Fe-S bond of P-450 may be modified from its normal orientation in model thiolates as the result of the constraints by the protein structure so as to lower the zero-field splitting constant.

2. (With Dr. Rispin and Dr. Sato) Continuation of #2 in 1976 report.

In the crystal field treatment of high spin Fe(III) complexes, only the two excited states, i.e. $^4T_1$ and $^2T_2$, have been customarily taken into account to explain the ground state ($^6A_1$) EPR properties. We have shown, however, that the lowest $^4T_2$ state derived from $t_2^4e$ configuration becomes increasingly important as the rhombicity of the crystal field increases. This is due to the fact that the axial as well as the rhombic components of the crystal field have non-zero off-diagonal matrix elements with $^4T_1$ state. Inclusion of the $^4T_2$ state enhances the calculated rhombicity of EPR g factors. We have formulated a complete scheme of direct matrix diagonalization and of calculation of g factors and the zero-field splittings to facilitate the investigation of the electronic state of high spin rhombic Fe(III) porphines.

3. (With Dr. Sato and Dr. Nebert) Continuation of #4 in 1975 report.

Use of EPR signals to estimate the relative amount of the high and low spin components in cytochrome P-450 has been further investigated to improve the quantitation. Inaccuracy in using the peak heights of the EPR signals as the measure of the high or low spin component arises from (1) the change of the signal shape from one system to another, (2) the observed signal does not represent the total amount in the case of high spin species because there are thermally populated three Kramers doublets (EPR signal originates from only the lowest one) and (3) the different temperatures required for observing the high spin ($\leq$10K) and the low spin ($\geq$50K) signal further complicates the intensity measurements. We have devised a simple method based upon the assumption that there is no spin state change as the result of the temperature change. The method does not require any corrections for the above factors and may be applied to study the effect of various in vivo as well as in vitro treatments on the relative amount of the two components.

4. (With Dr. Walter Lovenberg and Dr. G. A. Walker, NHLBI)

EPR study of a highly purified dopamine-β-hydroxylase (A) specimen (a copper containing enzyme, mediates the formation of norepinephrine from dopamines in the presence of ascorbate and oxygen) has shown that the binding of some nitrogen ligand to $Cu^{2+}$ ion takes place when substrates are added in the absence of $O_2$. In the presence of $O_2$, a protein-copper-oxygen complex appears to be formed. Also, the midpoint redox potential of (A) is estimated for the first time to be +310 mV using the EPR signal for titrations.

Short Term Future Plan:

EPR studies will be made of the high spin Fe(III) complexes, such as verdohemine, in which the porphine π-electron system has well-defined rhombic structure. Magnetic susceptibility measurement in Fe(II) high spin porphines and other complexes will start shortly. The fabrication of ENDOR system will continue.

Publications:

Sato, M., Rispin, A. S. and Kon, H.: EPR studies on high-spin Fe(III) tetraphenylporphine having rhombic character. Spin Hamiltonian and crystal field analysis of g values. Chem. Phys. 18: 211-224, 1976.

Sato, M., Kon, H., Kumaki, K. and Nebert, D. W.: Comparative EPR study on high spin ferric porphine complexes and cytochrome P-450 having rhombic character. Biochim. Biophys. Acta (in press).

Walker, G. A., Kon, H. and Lovenberg, W.: An investigation of the copper site(s) of dopamine-β-hydroxylase by EPR. Biochim. Biophys. Acta (in press).

Kumaki, K., Sato, M., Kon, H. and Nebert, D. W.: Correlation of type I, type II, and reverse type I difference spectra with absolute changes in spin state of hepatic microsomal cytochrome P-450 iron from five mammalian species. J. Biol. Chem. (in press).

Nebert, D. W., Kumaki, K., Sato, M. and Kon, H.: Association of type I, type II and reversed type I difference spectra with spin state of cytochrome P-450 iron. Proc. Third International Symposium on Microsomes and Drug Oxidation, Hamburg, Germany, 1976 (in press).

REPORTED IN PRESS - 1976

Rispin, A. S., Kon, H. and Nebert, D. W.: Electron spin resonance study of oxygen-17 enriched oxybenzo[a]pyrene radical. Mol. Pharm. 12: 476-482, 1976.

Sato, M., Kon, H., Akoh, H., Tasaki, A., Kabuto, C. and Silverton, J. V.: Anomalous magnetic properties of tetraphenylporphinato Co(II) complex in the solid state. Chem. Phys. 16: 405-410, 1976.

Weinbach, E. C., Harlow, D. R., Takeuchi, T., Diamond, L. S., Claggett, C. E. and Kon, H.: Aerobic metabolism of Entamoeba histolytica: facts and fallacies. Proceedings of the International Conference on Amebiasis, Sepulveda, B. and Diamond, L. S., (Eds.). Published by Instituto Mexicano del Seguro Social, Mexico, 1976, pp. 190-203.

| SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space) | U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT** | PROJECT NUMBER Z01-AM-29008-06-LCP |
|---|---|---|

PERIOD COVERED
July 1, 1976 to September 30, 1977

TITLE OF PROJECT (80 characters or less)
Electronic and molecular structural investigations

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: Ruth McDiarmid Research Chemist A LCP

COOPERATING UNITS (if any)

LAB/BRANCH
Laboratory of Chemical Physics

SECTION
Spectroscopy and Structure

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20014

| TOTAL MANYEARS: | PROFESSIONAL: | OTHER: |
|---|---|---|
| 1 | 1 | 0 |

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

1. The ultraviolet absorption spectrum of liquid ethylene was reinvestigated in an attempt to experimentally locate the origin of the $\pi \rightarrow \pi^*$ transition. 4 diffuse bands were observed whose transition energies appear to correspond with those extrapolated from bands observed at higher energy. The new experimental data supports the extrapolated position of the band origin.

2. The ultraviolet absorption spectra of $(SiH_3)_2S$, $[Si(CH_3)_3]_2S$ and $(GeH_3)_2S$ were measured and those of $[Si(CH_3)_3]_2O$ and $[C(CH_3)_3]S$ were remeasured to (a) aid in the analysis of the spectrum of $(CH_3)_2S$ and (b) investigate the nature of the Si-S bond. The new data suggests that one assignment in the old analysis of $(CH_3)_2S$ is incorrect.

3. The ultraviolet absorption spectra of trans-CHF=CHF and t-CDF=CDF were measured and analyzed. The old assignment of the spectrum of t-CHF=CHF is sho to contain inaccuracies. The first Rydberg transition leading to the second ionization potential of t-CHF=CHF is observed.

Project No: Z01-AM-29008-06-LCP

Project Description:

This research has two main objectives: To engender a better understanding of the electronic structure of molecules and to provide an experimental assessment of the progress of theoretical models of and calculations on molecules. To understand the structure of molecules, we seek to know, among other factors, the shape a given molecule assumes when the location(s) of its electrons(s) is altered, the effect the nature of the bonding group has on an electron not involved in that bond (inductive effect and/or indirect bonding effects) and the energy it requires to change the location of an electron. To attain these goals, we have been using electronic absorption spectroscopy because it directly provides information concerning the energies required to rearrange some of the electrons of a molecule and indirectly provides, through the analysis of the vibronic envelop of a transition, information concerning the change in shape of a molecule as some of the electrons are rearranged. By comparing the results obtained on different molecules, the effects of the nature of the bonding group is studied.

The investigations summarized above which have been conducted during the last year, for example, have shown that the best calculation yet attained for ethylene, a prototype molecule, does not adequately describe the experimental observations, especially in the vicinity of the equilibrium position of the first singlet excited state. The investigations have also shown that the substitution of a Si atom for a carbon atom in the sulfur ethers blocks or reduces the inductive effect of the $CH_3$ group.

In the next year we plan to continue this type of investigation. A recent calculation carried out on the molecule formamide has some interesting features that could bear experimental verification. We still plan to extend this type of investigation to unstable species, such as $H_2C{=}S$ and $HN{=}NH$, about which very little is experimentally known, although theoretical calculations have been conducted on these molecules. In addition, if the necessary equipment is purchased, we hope to apply this technique to cold molecules produced by a Joule-Thompson expansion through a small hole. Such molecules should have a simpler, hence less ambigious spectrum thereby allowing the experimental spectrum to be more precisely measured and more accurately analyzed. We hope also to move into the use of powerful, newer spectroscopic techniques, such as multiphoton absorption spectroscopy and saturation spectroscopy, which provide types of information not attainable from ordinary electronic absorption spectroscopy.

Publications:

REPORTED IN PRESS - 1976

McDiarmid, R.: Assignments in the electronic spectrum of $UF_6$. J. Chem. Phys., 65: 168-173, 1976.

| SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space) | U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT** | PROJECT NUMBER Z01-AM-29009-04-LCP |
|---|---|---|

PERIOD COVERED
July 1, 1976 to September 30, 1977

TITLE OF PROJECT (80 characters or less)
Studies on sickle cell disease

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

| | | | |
|---|---|---|---|
| PI: | William A. Eaton | Senior Surgeon | A LCP |
| OTHER: | James Hofrichter | Senior Staff Fellow | A LCP |
| | Frank Ferrone | Staff Fellow | A LCP |
| | Jon S. Gethner | Staff Fellow | A LCP |
| | Helen Sunshine | Guest Worker | A LCP |
| | Philip D. Ross | Research Chemist | A LMB |

COOPERATING UNITS (if any)

LAB/BRANCH
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SECTION
Spectroscopy and Structure

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20014

| TOTAL MANYEARS: | PROFESSIONAL: | OTHER: |
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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The thermodynamics and kinetics of the gelation of sickle cell hemoglobin are being investigated in order to develop an inhibitor of the reaction that could be used as a therapeutic agent.



PHS-6040
(Rev. 10-76)

Project No: Z01-AM-29009-04-LCP

Objectives:

The major aim of this work is to elucidate the molecular mechanism of hemoglobin S gelation in order to provide a rationale for the development of agents that can be used in the treatment of patients with sickle cell disease.

Methods Employed:

In addition to conventional biochemical and physical methods a variety of specially-designed techniques have been employed to study the gelation reaction. These include microspectrophotometry, linear dichroism and birefringence, turbidity, water proton NMR linewidths, scanning microcalorimetry, temperature perturbation kinetics, and laser-induced photolysis.

Major Findings:

I. Thermodynamics of Deoxyhemoglobin S Gelation

This study was designed to obtain the basic thermodynamic parameters for the gelation of deoxyhemoglobin S by measuring the heat of the reaction using scanning calorimetry and the solubility in a high speed sedimentation experiment.

The solubility was measured over the temperature range 15-45°C. The van't Hoff enthalpy change calculated from this data in the ideal approximation is in close agreement with the observed heats determined from scanning microcalorimetry. There is a large apparent negative heat capacity change of -197 cal/deg-mole.

To analyze these thermodynamic results our previous two-phase (crystalline polymer plus monomer solution), two component (deoxyhemoglobin S plus solvent) model for gelation has been extended to include the effects of solution non-ideality. Osmotic pressure and sedimentation equilibrium studies by others indicate that a large contribution to the activity of hemoglobin in the solution phase results from the geometric effect of excluded volume. Incorporating the solution phase non-ideality in the calculation of van't Hoff enthalpies implies that the close agreement with the calorimetrically-determined enthalpy changes is fortuitous. To obtain standard state enthalpy changes, i.e. the values at infinite dilution, the van't Hoff enthalpies must be corrected for the temperature dependence of the activity coefficient, while the calorimetric enthalpies must be corrected for heats of dilution. Neither of these quantities has yet been measured. In spite of these uncertainties in the $\Delta H°$, approximate standard state free energy and entropy changes can be obtained at 37°C, where $\Delta H \approx 0$, and the reaction is entirely entropically driven. These are $\Delta G° = -3$ kcal/mole and $\Delta S° = 9.6$ e.u. These quantities provide important constraints on any theoretical model for the bonding between deoxyhemoglobin S molecules in the polymer, and the interaction between polymers that leads to their alignment.

II. Kinetics of Deoxyhemoglobin S Gelation

The object of this study is to obtain experimental kinetic parameters that can be used to test models on the detailed molecular mechanism of the gelation reaction.

Previous measurements by us on the concentration and temperature dependence of deoxyhemoglobin S gelation kinetics employed the linear birefringence technique, which detects the formation of the aligned polymer phase. The reaction may also be monitored be measuring the turbidity. The turbidity technique affords greater precision in sample preparation and the ability to obtain thermodynamic data on the same sample in sedimentation experiments, while the birefringence technique permits investigation of the reaction on shorter time scales.

Using the turbidity technique the concentration and temperature dependence of the delay time were measured. Both the apparent high order ($\sim$35) and high apparent activation energy were found to be the same as was previously obtained with the birefringence technique. Thus either turbidity or birefringence measurements may be employed to obtain kinetic parameters for the reaction.

We have previously proposed that the rate of gelation is determined by the formation of thermodynamically unstable nuclei. In order to test such a nucleation model it is necessary to measure the number concentration of polymers as a function of time. With the present techniques only the total concentration of polymerized hemoglobin can be measured. We have initiated a series of temperature-jump experiments which have the possibility of kinetically counting the polymer concentration. The basic idea of these experiments is that in a gelled sample at equilibrium or in a sample where extensive polymerization has taken place, the solution phase is not sufficiently supersaturated for the nucleation of more polymers to take place. The application of a small temperature perturbation will therefore result in the addition or subtraction of monomers from pre-existing polymers. The rates of these processes will be proportional to the concentration of polymers.

Temperature-jump experiments on gelled samples near quilibrium were carried out on samples of the same concentration and at the same temperature, where the delay time was varied by changing the initial gelling temperature. Two distinct relaxation rates were observed in birefringence experiments. The faster rate probably reflects polymerization and depolymerization, while the slower rate may arise from the reordering of polymers in the aligned phase. The rate of the fast process increases with decreasing delay time, suggesting that samples gelled with shorter delay times do indeed contain a higher concentration of polymers, even though the total amount of polymerized hemoglobin is known from sedimentation experiments to be identical. This result is in agreement with the qualitative prediction of a nucleation model.

III. Photolysis of Carbonmonoxyhemoglobin S

This study is designed to obtain kinetic results on very short time scales and in the presence of ligand binding.

All of the techniques that have been employed thus far have been limited to times greater than about 30 seconds, the time necessary to produce the large temperature changes that induce the gelation reaction. Kinetic studies by others on red cells have shown, however, that gelation can take place in times as short as 0.03 second. Gelation within red cells inside capillaries of patients, moreover, can also take place in the sub-second range. In order to study the gelation reaction in the sub-second range, polymerization can be rapidly induced by the photolysis of carbonmonoxyhemoglobin S. Removal of the carbon monoxide affords a flexible and rapid means of controlling the deoxyhemoglobin concentration, and thereby the rate of gelation. The photolysis technique can also be used to investigate questions concerning the rate of polymerization and depolymerization in already gelled samples (see section II), as well as the binding of carbon monoxide by the polymer. Here the newly-developed technique of excitation-modulation spectroscopy is used, employing the reversibility of carbon monoxide photodissociation to make small, periodic perturbations on the gelled sample. By altering the ligand-hemoglobin equilibrium, the monomer-polymer equilibrium is also altered. The advantage of the modulated exciation is that the changing ligand-hemoglobin equilibrium can be nulled to permit the direct, sensitive monitoring of the monomer-polymer process. This method will also extend the sensitivity with which liganded material incorporated into the polymer can be measured, by coupling modulated excitation with linear dichroism measurements. These experiments can lead to carbon monoxide binding curves for the polymer.

The efforts to date have been directed at assembling the instrumentation required to carry out the above experiments. An argon ion laser recently acquired will be used as the photolysis source.

IV. Molecular Mechanism of Gelation

This study is aimed at providing a model for the molecular mechanism of gelation that makes quantitative predictions of the kinetic behavior of the system, and yields parameters that can be interpreted in terms of molecular structure. Such a model consists of a series of coupled differential equations that describe the nucleation, growth, and alignment of polymers. Previous theoretical studies have been limited to treatments that yield analytic expressions for the rate of polymer formation. Such treatments require simplifying assumptions that can only be justified by numerical analysis.

We have begun such an analysis of our nucleation model by numerically integrating series of coupled differential equations on the DEC10 system. An adequate model may require the solution of over 50 simultaneous differential equations, since experimental data suggest that the nucleus contains at least 15 molecules, and polymers contain up to thousands of molecules.

Thus far calculations have only been carried out for the formation of a 10-mer, with nuclei containing from 4 to 9 molecules. The results suggest that: (i) the steady state assumption for all intermediates, as postulated in our original kinetic model, is an excellent approximation, except for the very initial stages of the reaction; (ii) the more restrictive assumption that the nuclei are in continuous equilibrium with monomer may be approximately obeyed, permitting an enormous simplification of the theoretical treatment if found to be generally valid; (iii) the apparent order of the reaction, as given by the concentration dependence of the delay time, is about half of the number of molecules in the nucleus, and (iv) a pure nucleation model may not be capable of explaining the highly autocatalytic progress curves, suggesting that an additional process must be incorporated into the mechanism.

V. Gelation of Deoxyhemoglobin S Mixtures

The object of this study is to describe the kinetics and thermodynamics of the gelation of hemoglobin S in mixtures with other hemoglobins, and thereby provide quantitative criteria for the amount of inhibition of the gelation reaction that will be required to obtain a therapeutic effect in patients with sickle cell disease.

It has been known for some time that patients with high hemoglobin A or F content exhibit decreased clinical severity. For example, patients with sickle cell trait (60% HbA, 40% HbS) are symptom-less, patients with hereditary persistence of fetal hemoglobin (30% HbF, 70% HbS) have a much milder form of sickle cell disease, and patients with the double heterozygous condition, sickle-$\beta^+$-thalassemia (15-30% HbA, 70-85% HbS) have somewhat decreased clinical severity. If we accept the hypothesis that the delay time for gelation is the primary determinant of clinical severity, then kinetic studies on the above mixtures should provide the quantitative criteria that we are seeking.

A preliminary set of kinetic and solubility measurements have been carried out on mixtures where the percentage of HbA or HbF has been varied from 0 to 30%. At 30% HbA or HbF, the delay time is lengthened by about a factor 2000-3000. This result suggests that for an agent to have a clear therapeutic effect, a several thousand-fold increase in the delay time may be necessary. None of the non-covalent inhibitors of gelation that have been proposed thus far are known from kinetic experiments or predicted from theoretical calculations using empirical relations, to increase the delay time by even a factor of 100 at concentrations that can be tolerated by humans. To obtain the required increase is still theoretically possible, however, since solutions that are 30% saturated with carbon monoxide exhibit delay times that are increased by a factor of 1000. Also, if some means were found to decrease the intracellular hemoglobin concentration by about 20%, a several thousand-fold increase in the delay time should result.

The solubility measurements on these mixtures are useful for understanding the mechanism of inhibition of the additional hemoglobin component (HbA or HbF). Non-ideality in these concentrated solutions appears to play a crucial

role. Almost all of the increase in the solubility resulting from the addition of hemoglobin A or F can be explained by excluded volume non-ideality theory, i.e. the added hemoglobin occupies space in the solution that is otherwise available for the hemoglobin S.

Significance to Biomedical Research and the Program of the Institute:

Our kinetic studies constitute a totally new approach to understanding the pathophysiology of sickle cell disease and to the development of therapeutic measures.

The theoretical approaches, the experimental results, and the methods developed in this investigation should contribute to a general understanding of other macromolecular self-assembly processess.

Proposed Course of Project:

Temperature perturbation experiments will be carried out to obtain kinetic parameters that are necessary to test and refine models for the molecular mechanism of hemoglobin S gelation. Laser photolysis experiments on carbonmonoxyhemoglobin S solutions will be carried out to study the gelation reaction on short time scales, and to mimic in vivo gelation. Theoretical models for the molecular mechanism of gelation will be explored using numerical techniques. Further experiments on hemoglobin mixtures will be performed in order to more precisely define the amount of inhibition of gelation that will be required to obtain a therapeutic effect in patients. The kinetics and thermodynamics of gelation will be studied in solutions partially saturated with oxygen.

Publications:

Ross, P.D., Hofrichter, J. and Eaton, W. A.: Thermodynamics of gelation of sickle cell deoxyhemoglobin. J. Mol. Biol., in press.

REPORTED IN PRESS - 1976

Hofrichter, J., Ross, P. D. and Eaton, W. A.: Supersaturation in sickle cell hemoglobin solutions. Proc. Nat. Acad. Sci. 73: 3035-3039, 1976.

Hofrichter, J., Ross, P. D. and Eaton, W. A.: A physical description of hemoglobin S gelation. In Hercules, J. I., Cottam, G. L., Waterman, M. R. and Schecter, A. N. (Eds.): Proceedings of the Symposium on Molecular and Cellular Aspects of Sickle Cell Disease. DHEW Publication No. (NIH) 76-1007, 1976, pp. 185-223.

| SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space) | U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT** | PROJECT NUMBER Z01-AM-29010-05-LCP |
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PERIOD COVERED
July 1, 1976 to September 30, 1977

TITLE OF PROJECT (80 characters or less)

Conformation and electronic structure of biological molecules

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

| | | | |
|---|---|---|---|
| PI: | William A. Eaton | Senior Surgeon | A LCP |
| OTHER: | Louise K. Hanson | Guest Worker | A LCP |

COOPERATING UNITS (if any)

LAB/BRANCH
Laboratory of Chemical Physics

SECTION
Spectroscopy and Structure

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20014

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The electronic structure of heme proteins is being investigated using a combined theoretical and experimental approach, that includes extended Hückel molecular orbital calculations and various electronic spectroscopic measurements.



PHS-6040
(Rev. 10-76)

Project No: Z01-AM-29010-05-LCP

Objectives:

The aim of this research is to relate the stereochemistry and electronic structure of biological molecules, particularly proteins with chromophoric prosthetic groups, to their biochemical reactivity and function.

Methods Employed:

In addition to conventional biochemical and spectroscopic methods, techniques have been developed for measuring anisotropic optical properties on microscopic samples. Dr. Hofrichter has designed and constructed a general phase modulation microspectrophotometer which is capable of measuring linear dichroism, circular dichroism, linear birefringence, and circular birefringence with sensitivity comparable to commercial instruments used for macroscopic samples. The instrument also measures direct polarized absorption, and is interfaced to our Honeywell 516 computer.

In order to interpret the electronic spectra in terms of the orbital promotion mechanism responsible for observed electronic transitions we have used the self consistent charge, extended Hückel LCAO-molecular procedure developed by Gouterman and coworkers at the University of Washington.

Major Findings:

I. Electronic structure of oxy-, deoxy-, and carbonmonoxyhemoglobin:

Extended Hückel calculations were performed on imidazole-Fe-$O_2$ porphin, imidazole-Fe-CO porphin, and imidazole-Fe porphin complexes using atomic coordinates derived from small molecule and heme protein x-ray studies. These complexes represent the chromophores of the corresponding heme protein derivatives. The geometry of binding of imidazole, $O_2$ and CO, the iron out-of-plane displacement, and the degree of doming of the porphin ring were systematically varied in order to study the effects of stereochemistry on the orbital energies and wavefunctions. Transition frequencies were obtained from orbital energy differences after correction for electron repulsion and exchange using crystal field parameters, where possible. In the case of the model complex for deoxyhemoglobin, these corrections are as large or larger than the orbital energy differences for the low frequency transitions. The results of these calculations have been used as a guide in making spectroscopic assignments of the low frequency transitions of oxy-, deoxy-, and carbonmonoxyhemoglobin that are consistent with the observed polarized single crystal absorption, natural circular dichroism, and magnetic circular dichroism spectra.

II. Cytochrome P-450

Polarized single crystal absorption spectra of imidazole-bound ferric P-450 and substrate-free ferric P-450 from the bacterium P. putida have been measured. Two weak near infrared bands in the imidazole ferric P-450 spectrum, which also occur in the mercaptide complexes of ferric myoglobin, were found to be polarized perpendicular to the porphin plane, indicating

that they originate from charge-transfer type transitions. The similarity of the mercaptide-myoglobin and imidazole-P450 spectra suggest that sulfur is an axial ligand for the iron in P-450.

Significance to Biomedical Research and the Program of the Institute:

The elucidation of the electronic structure of the active sites of proteins should lead to a much deeper understanding of how these molecules perform their biological function.

The techniques developed in our investigations may be applied to a wide variety of problems in protein structure and chemistry.

Proposed Course of Project:

Magnetic and natural circular dichroism measurements on hemoglobin derivatives will be carried out to as low a frequency as possible in order to establish the nature of the lowest electronic states. The results of the theoretical and spectroscopic studies will be used to interpret spectral perturbation studies, in order to assess the influence of the protein on the electronic structure and conformation of the heme.

Publications:

Hanson, L. K., Sligar, S. G. and Gunsalus, I. C.: Electronic structure of cytochrome P450. Croatia Chemica Acta 2: 237-250, 1977.

REPORTED IN PRESS - 1976

Eaton, W. A., Hofrichter, J., Hanson, L. K. and Makinen, M. W.: Single crystal optical spectroscopy of hemoglobin. In Kotani, M. and Tasaki, A. (Eds.): Metalloprotein studies utilizing paramagnetic effects of the metal ions as probes. Osaka University, Japan, 1975, pp. 51-88.

Hofrichter, J. and Eaton, W. A.: Linear dichroism of biological chromophores, Annual Review of Biophysics and Bioengineering 5: 511-560, 1976.

| SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space) | U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT** | PROJECT NUMBER ZO1-AM-29011-06-LCP |
|---|---|---|

PERIOD COVERED
July 1, 1976 through September 30, 1977

TITLE OF PROJECT (80 characters or less)

The Physics and Chemistry of Photoreception

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

| | | | |
|---|---|---|---|
| PI: | William A. Hagins | Medical Officer | A LCP |
| OTHER: | W. E. Robinson | Senior Staff Fellow | A LCP |
| | S. Yoshikami | Guest Worker | A LCP |
| | F. M. Hagins | Guest Worker | A LCP |
| | J. Meisner | Laboratory Technician--Summer Employee 6 June-30 September 1977 | A LCP |

COOPERATING UNITS (if any)

None

LAB/BRANCH
Laboratory of Chemical Physics

SECTION
Section on Membrane Biophysics

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20014

| TOTAL MANYEARS: | PROFESSIONAL: | OTHER: |
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| 4 | 3 | 0.4 |

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

An investigation of the mechanism of visual excitation in vertebrates.



PHS-6040
(Rev. 10-76)

Project Description:

The central aim of this project is to describe the chemical and electrical steps that connect absorption of a photon of visible light in a photoreceptor cell with the transmission of a sensory signal across its synapse with the distal neurons of the brain. Previous work by our group on the rod and cone cells of vertebrate retinas have established that an essential part of their excitatory machinery is a large ionic current that circulates through these cells in darkness. This "dark current" enters the plasma membrane covering the light absorbing outer segment of a rod or cone, passes down the cytoplasm of the cell and emerges into the extra-cellular space through the plasma membrane of its more proximal parts. Light absorbed in the outer segment quickly and reversibly reduces the dark current, thus hyperpolarizing the cell and probably changing the rate of release of a neurosensory transmitter substance at its synapse with deeper lying neurons.

During the period 1 July 1976 through 30 September 1977 our group has continued to study (1) the question of the identity of the sensory transmitter substance conveying excitation from the intracellular disks of rod outer segments to the overlying plasma membrane and (2) the biochemical basis of the transmitter cycle and the source of free energy that drives it.

Question (1) Although there is much indirect evidence that $Ca^{++}$ ions are the sensory transmitter substance, we are attempting to demonstrate this directly with the aid of a $Ca^{++}$-sensitive azo dye that can be introduced into retinal cells by the phospholipid vesicle fusion technique. The dye, dichlorophosphonazo III has been studied (Yoshikami & Hagins) and found to be an indicator for $Ca^{++}$ (1 micromolar dissociation constant $K_D$ at pH 7), $Mg^{++}$ ($\sim$1 mM $K_D$) as well as a pH indicator. Computer analysis of families of absorption spectra disclose that, once in the cells, it is capable of simultaneously reporting changes in $[Mg^{++}]$ $[Ca^{++}]$ and $[H^{+}]$. A multichannel spectrometer has been built for performing such analyses on thin tissue layers such as retinas (Rashidian, Yoshikami, Hagins). Tests show that it has the predicted sensitivity for the relevant ions and can follow transient absorbance changes of $\sim 10^{-4}$A with millisecond time resolution. Experiments with live retinas are under way.

Question (2) Studies of the nucleotide phosphate metabolism of isolated frog retinal rod outer segments have continued (Robinson & Hagins). An apparently new enzyme has been discovered in them. It is a guanosine triphosphatase that splits GTP to GDP and Pi. Although inactive in darkness, it becomes fully active within less than 200 msec after illumination and can be half-activated by light exposures that bleach less than 1 rhodopsin molecule in 2000. Thus the enzyme is activated by rhodopsin but is not rhodopsin itself. The activated enzyme splits more than 10 GTP's per second per bleached rhodopsin, thus providing in principle a source of chemical free energy greatly exceeding that available from the absorbed photons. The connection between the enzyme and $Ca^{++}$ metabolism has not yet been established, but it is likely to be an important link in visual excitation, since it is at present the only known enzyme rapidly activated by light in retinal rods.

Additional Project:

Because the study of the biochemistry and physiology of visual responses requires ways to rapidly preserve tissues in states close to life, we have begun an investigation of fast-freezing techniques. Using high-speed cinematography and a specially made fast sapphire-block freezer (J. Meisner, M. Rashidian), the propagation of ice at -190C into supercooled biological samples has been observed. During this process, solutes in cell cytoplasm are redistributed. Methods are now being developed to study this redistribution quantitatively.

Our studies indicate that retinal rods and cones share much of the electrical and biochemical machinery of neurons. Their most conspicuous difference is their huge dark current, which turns over their entire content of univalent cations in about 1-5 minutes and requires a rate of metabolism as high as that of any known tissue. Light, in controlling this current, not only acts as a sensory stimulus but also must have a great effect on the metabolic load that rods and cones carry. A clear understanding of the metabolism of these cells is therefore important in handling certain retinal vascular diseases and retinal detachment. The scientific importance of understanding the mechanism of visual excitation speaks for itself.

Publications:

Weinstein, J. N., Yoshikami, S., Henkart, P., Blumenthal, R. and Hagins, W. A.: Liposome-Cell Interaction: Transfer and Intracellular Release of a Trapped Fluorescent Marker, Science, 195, 489-492 (1977).

Hagins, W. A. and Yoshikami S.: Intracellular Transmission of Visual Excitation in Vertebrate Retinal Photoreceptors: Electrical Effects of Chelating Agents Introduced into Rods by Vesicle Fusion, Rank Prize Fund Symposium on Photoreception, London, 1976, H. B. Barlow and P. Fatt, eds. New York, Academic, in press.

| SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space) | U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT** | PROJECT NUMBER Z01-AM-29012-07-LCP |
|---|---|---|

PERIOD COVERED
July 1, 1976 through September 30, 1977

TITLE OF PROJECT (80 characters or less)

The influence of molecular structure on chemical and biological properties

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

| | | | |
|---|---|---|---|
| PI: | Norman E. Sharpless | Research Chemist | A LCP |
| OTHER: | Ralph G. Adams | Research Physicist | A LCP |
| | Frederick S. Brackett | Scientist Emeritus | A LCP |
| | William H. Jennings | Research Physicist | A LCP |
| | William M. Egan | Guest Worker | A LCP |
| | Peter F. Kador | Visiting Fellow | EI LVR |

COOPERATING UNITS (if any)
C. H. L. Kennard — Reader in Chemistry, University of Queensland, Brisbane, Australia

LAB/BRANCH
Laboratory of Chemical Physics

SECTION
Section on Membrane Biophysics

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20014

| TOTAL MANYEARS: | PROFESSIONAL: | OTHER: |
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| 1.0 | 1.0 | 0.0 |

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords) Energy minimization calculations on retinal isomers gave quantitative explanations to the disorder in the cyclohexene ring, showing energetics of interconversion consistent with the crystal structure uncertainty and lack of NMR resolution, as well as confirming the known order of stability and extending it to other conformers. The sources of non-planarity and energy differences in the conformers of retinal are due to non-bonded interactions within the molecule. $^{13}C$ NMR spectra of DDT and analogs have been obtained for correlation with X-ray structural data. The NMR results have been investigated with respect to quantum parameters, Taft and Hammett functions. Energy minimization calculations show the order of stability to be m,p-DDT > p,p-DDT > o,p-DDT >> the 1,1,1-tribromo analog. Comparison of their stability energies with reaction rate constants (dehydrohalogenation) and the $^{13}C$ NMR clearly indicate the large important steric effect due to the chlorine atom in the ortho position. The inhibition, by flavonoids, of aldose reductase, thought to be involved in the formation of sugar cataracts has been investigated by molecular orbital methods. The data show the efficiency of aldose reductase inhibition depends on the energy of the lowest empty molecular orbital of the flavonoids, and results from a nucleophilic attack on the carbonyl carbon atom.

Project Description:

This project investigates the molecular and submolecular factors, such as structural features, quantum parameters and physical properties in relation to biological and pharmacological behavior of biologically important substances.

1. Energy minimization.

This computational method is an extremely powerful approach to biological structural problems in that it gives the conformation and energy of the most stable forms, whereas the previously utilized quantum methods can only give the energy of selected conformations, requiring a trial and error approach to conformation studies. This is extremely important in our current series of the retinal isomers, in which the molecular properties are solely conformational. Calculations by this method on the various isomers of retinal, (as well as some not yet described)-(ie. all-trans, all cis) by this strain energy minimization technique show that the stabilities of the various isomers depend almost solely on non-bonded interactions. The known limited stability series of several retinal isomers has been confirmed, and also expanded to include other isomers. The calculations confirm the non-planarity around the $C_6$-$C_7$ bond, and show also that in 11-cis retinal the $C_{11}$-$C_{12}$ bond is out-of-plane. Certain valence angles are distorted due to van der Waals interactions.

The 'disorder' in the cyclohexene ring, which has prevented both precise crystal structure data and NMR resolution is due to the small energy difference between the two possible half-chair conformations of only 0.067 kcals/mol. This extremely low interconversion barrier indicates a 53-47 ratio between the two conformers, and a very high rate of interconversion.

Two other possible conformers of the cyclohexene ring, both boat forms are less stable than the chair forms by about 10 kcals/mol.

The program is now being modified to permit it to connect directly with the graphics system X-ray, and preliminary molecular sketches have been obtained.

The results of spectral investigations of the 1-α-acetyl lysyl-1-alanyl-1-phenyl alanine methyl ester/retinal complex as a model of the visual protein opsin, will be found in Dr. Adams' report.

The $^{13}C$ NMR spectra and the structures and energies of DDT and analogous compounds have been obtained in conjunction with X-ray crystallographic data being measured by Dr. Colin Kennard, University of Queensland, Brisbane. A total of fifteen compounds has been investigated, based on modifications in the original DDT molecule: 1) Changing the position of the chlorine atom on one ring. 2) Removal of the chlorine atoms from the trichloromethyl group. 3) Variations in the substituents on the aromatic ring and 4) miscellaneous derivatives of DDT. The data have been analyzed in terms of quantum chemistry parameters and Taft or Hammett functions.

Calculations of conformations and energies of p,p; o,p, m,p-DDT and the 1,1,1-tribromo compound shows the m,p-DDT to be the most stable, followed in

order by p,p-DDT, o,p-DDT and the tribromo derivative, which is by far the least stable. Comparing the stabilities thus calculated with the known reaction rate constants for dehydrohalogenation shows the rate constant to decrease with stability, save for the o,p-DDT. This reflects the steric effects of the chlorine atom at the ortho position. Comcommitant with this is the upfield shift of the α-carbon atom (ca 5.7 ppm), the presumptive site of the attack by the hydroxide ion.

Aldose reductase has been implicated in the events which lead to sugar cataracts by its action in forming sugar alcohols, possible causes of the cataracts. Flavonoids have been reported to be potent inhibitors of aldose reductase. Screening of a series of flavonoids for aldose reductase inhibition showed a statistically significant relationship between inhibition and the energy of the lowest empty molecular orbital. The preferred site of the reaction, as calculated, is the carbon atom of the carbonyl group, indicating that the mechanism of inhibition is a nucleophilic assault on the carbonyl atom of the flavonoid by the reductase, which then becomes unavailable to its normal substrate.

There are other carbonyl compounds which show reductase inhibition, but the consistency and generally higher efficiencies of the flavonoids indicates that certain stereochemical requirements must also be met.

Cyclophosphamide, an anti-cancer drug, contains a six-membered hetero cyclic ring capable of existing in 4 different conformers. Complete energy minimization calculations are at present being carried out on these forms.

| SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space) | U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT** | PROJECT NUMBER Z01-AM-29013-06-LCP |
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PERIOD COVERED

July 1, 1976 through September 30, 1977

TITLE OF PROJECT (80 characters or less)

The physics and chemistry of the visual pigments

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

| | | | |
|---|---|---|---|
| PI: | Ralph G. Adams | Research Physicist | A LCP |
| OTHER: | Norman E. Sharpless | Research Chemist | A LCP |
| | William H. Jennings | Research Physicist | A LCP |

COOPERATING UNITS (if any)

None

LAB/BRANCH

Laboratory of Chemical Physics

SECTION

Section on Molecular Biophysics

INSTITUTE AND LOCATION

NIAMDD, NIH, Bethesda, Maryland 20014

| TOTAL MANYEARS: | PROFESSIONAL: | OTHER: |
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☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Model systems using retinal and retinal isomers in π-π complex with a tripeptide are being investigated as approximating the visual pigment, rhodopsin.



PHS-6040
(Rev. 10-76)

Project Description:

The primary purpose of this project continues to be the eluciation of structure and function of the chromophore (retinal) of the mammalian pigment rhodopsin. Ideally the resultant data will contribute to construction of an *in vitro* system which will approximate the function of retinal *in vivo*.

We assume that the manner in which retinal functions will depend strongly on its structure and its milieu. Currently we are concluding a series of experiments (data are primarily from visible spectra) concerned with close neighbor effects. This consists of subjecting the $\pi$-$\pi$ complex formed between retinal and a tripeptide ($\alpha$-acetyl-l-lysyl-l-alanyl-l-phenylalanine methyl ester) to solutions of various amino acids and derivatives in various organic solvents. The result thus far is that only phenylalanine seems to be effective in changing the maximum absorption of the $\pi$-$\pi$ complex although still does not shift to the desired 500 nm. The best combination thus far is 492 nm. All the possible combinations have not been exhausted.

The National Research Council recently asked for additional data on retinal and its isomers lending added importance to our current computer generated data on the structure and energy of retinal isomers. (The program is discussed by Dr. Sharpless in his report.) The data aids in narrowing the area of investigation of isomers of retinal as candidates for intermediates in the bleaching of rhodopsin as proposed in current literature. To date we have only verified the possibility that 11-13 di-cis retinal could be an intermediate by virtue of its configuration and energy requirements to isomerize to 11-cis retinal.

Future Work:

1. Organization of data currently existing into publishable form from computer storage.

2. Continuation of above lines of research which are only now yielding publishable data.

3. Should above efforts be completed before end of the year several protein syntheses are desirable to build a surround for the $\pi$-$\pi$ complex described above.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (do NOT use this space)

U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE
PUBLIC HEALTH SERVICE
NOTICE OF
INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01-AM-29014-17-LCP

PERIOD COVERED

July 1, 1976 through September 30, 1977

TITLE OF PROJECT (80 characters or less)

Effects of environmental stress (hypoxia, exercise, smoke) on animals.

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

| | | | |
|---|---|---|---|
| PI: | Paul D. Altland | Research Physiologist | A LCP |
| OTHER: | Milton G. Parker | Biologist | A LCP |

COOPERATING UNITS (if any)

Barnett Rattner (Pre-doctoral Guest Worker)
Department of Zoology, University of Maryland

LAB/BRANCH

Laboratory of Chemical Physics

SECTION

Section on Physiology

INSTITUTE AND LOCATION

NIAMDD, NIH, Bethesda, Maryland 20014

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

In a study of the effects of graded concentrations of ambient cigarette smoke on systemic changes in mildly exercised and resting rats, hypothermia was induced only in exercised rats with 15 mg/m$^3$ suspended particulate matter (SPM) and 13 ppm carbon monoxide (CO). Plasma lactic dehydrogenase (PLDH) concentration was elevated after exercise in smoke containing 35 mg/m$^3$ SPM and 51 ppm CO. An increase in concentrations of plasma creatine kinase and blood glucose and in the density of PLDH isoenzyme Band 5 were found in exercised and resting rats at the highest smoke level studied (199 mg/m$^3$ SPM and 220 ppm CO). Plasma corticosterone was elevated only after exercise at the 2 highest smoke levels. The amount of cigarette smoke needed to induce significant systemic changes in rats greatly exceeded acceptable National air quality standards and threshold limits for SPM (National 0.075 mg/m$^3$, American Conference of Governmental Industrial Hygienists, 10 mg/m$^3$) and for CO (National 9 ppm, ACGIH 50 ppm) established for man.



PHS-6040
(Rev. 10-76)

Project No.: Z01-AM-29014-17-LCP

Project Description:

To determine physiological, biochemical and pathological changes which occur following exposure to altitude (hypoxia), exercise and other environmental stresses. Altitude exposures were conducted in a small decompression chamber or in a chamber through which selected $O_2$-$N_2$ gas mixtures were circulated. Exercise was performed in an exercise drum located within a chamber designed to circulate cigarette smoke.

Recently the possible deleterious effects of atmospheric pollutants on man have received wide publicity. Reports on the effects of cigarette smoke induced by smokers in a confined space or by smoking machines in chambers have shown increased incidence of subjective symptoms such as headaches, irritation of eyes and respiratory passages, but relatively little is known of systemic effects of various concentrations of ambient cigarette smoke on man or animals. Exposure to environmental stresses such as hypoxia or exercise have produced changes in blood chemistry and body temperature and weight, that aided in the diagnosis of the severity of stresses. No such studies have been previously conducted with ambient cigarette smoke. Male rats were exposed to cigarette smoke concentrations from 2.4 $mg/m^3$ suspended particulate matter and 8 ppm carbon monoxide to 199 $mg/m^3$ SPM and 220 ppm CO for 4 hours at rest or after light intermittent exercise. Hypothermia (37.3°C) was produced after exercise in smoke containing 15 $mg/m^3$ SPM and 13 ppm CO, and its severity increased at higher smoke concentrations. Plasma lactic dehydrogenase increased significantly after exercise in smoke containing 34 $mg/m^3$ SPM and 51 ppm CO. An increase in the concentration of plasma creatine kinase and of blood glucose, and in the density of PLDH isoenzyme BAND 5, were found only at the highest level of smoke (199 $mg/m^3$ SPM and 220 ppm CO). Plasma corticosterone concentrations were elevated after exercise in the two highest smoke concentrations. No tissue changes were found, so elevations in plasma enzymes were attributed to increased cellular permeability. Some changes induced by smoke were more severe following light exercise and were attributed to SPM at levels exceeding 34 $mg/m^3$ and to tissue hypoxia (COHb 11.7%) induced by CO concentrations above 51 ppm. The amount of cigarette smoke needed to induce significant systemic changes in rats greatly exceeded acceptable National air quality standards and threshold limits for SPM and CO established for man.

Experiments are being conducted on the effects of several of the major components of ambient cigarette smoke such as carbon monoxide, particulate matter and nicotine on systemic and bodily changes in rats. Work will continue on the effects of hypoxia (simulated altitude) on male endocrine and reproductive systems of mice using histological and competitive protein binding radioimmunoassay techniques (with guest worker Barnett Rattner). Exploratory studies are being conducted on the possible usefulness of anti-anginal drugs in altering the altitude tolerance of mice and rats.

Publications:

Altland, P. D.: Altitude and exercise effects on animals: In: Progress in Biometeorology Division B, Progress in Animal Biometeorology Vol. 1, Pt. 2,

Chapter 3, pp 124-131, 1963-1973, Ed. H. D. Johnson, Swets and Zeitlinger, Amsterdam, 1976.

Rattner, B. A., Ramm, G. M. and Altland, P. D.: Effects of hypoxic exposure on embryonic implantation in mice. Proc. Soc. Exp. Biol. Med. 153: 138-142, 1976.

Altland, P. D., Parker, M. G. and Brubach, H. F.: Effects of graded concentrations of cigarette smoke on plasma enzyme, corticosterone, and body temperature. Environmental Research (in press).

| SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space) | U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT** | PROJECT NUMBER Z01-AM-29015-06-LCP |
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PERIOD COVERED
July 1, 1976 through September 30, 1977

TITLE OF PROJECT (80 characters or less)

Digital computer facilities for LCP and LMB

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: William H. Jennings, Jr. Research Physicist A LCP
OTHER: None

COOPERATING UNITS (if any)

Computer Systems Laboratory, DCRT

LAB/BRANCH
Laboratory of Chemical Physics

SECTION
Section on Membrane Biophysics

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20014

| TOTAL MANYEARS: | PROFESSIONAL: | OTHER: |
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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The laboratory computer facility serving LCP and LMB was available for data acquisition during 96% of the reporting period. The trend to more extensive data processing continues; however, system availability can be maintained by the partitioning of some of the data acquisition load onto dedicated microcomputer systems (now in development) and some of the more extensive data processing load onto the DEC-10 central facility.



PHS-6040

Project No.: Z01-AM-29015-06-LCP

Project Description:

The demands upon this laboratory computer facility have often exceeded capacity during the past two years and hardware changes in the system have been anticipated. However, small computer hardware has been one of the most rapidly changing technologies during this period and a deliberate approach to system changes is essential. In cooperation with the Computer Systems Laboratory of DCRT, we are developing a dedicated micro-computer data acquisition system which will occupy the first level in a three level hierarchical computer system. On the second level will be the present Honeywell system linked with a micro computer communications front end by a high speed parallel channel. The interchange of data between the remote dedicated systems on level one and the communications system at level two will be by serial lines using an industry standard line protocol. At level three will be the DEC-10 central facility and the link to it will be via a high speed serial line using the same protocol as that between levels one and two. Two prototypes of the level one system are anticipated; the first is scheduled to begin tests on or before 1 Jan. 1978. These hardware changes necessitate the addition of a high speed direct memory channel to the Honeywell mainframe and a reconfiguration of the operating system.

Objectives:

Development of the computer hardware and software elements used for real-time data acquisition from laboratory instrumentation and for the management and processing of this data.

Findings:

The most recent addition to this system, the EPR (electron paramagnetic resonance) was brought into routine operation after major changes in the data acquisition software and modifications to the instrument interface electronics. Revisions of data processing programs were made to accommodate the EPR, to utilize new sub-programs and to enhance user-program interaction, specifically where previous logic mislead the user or permitted serious errors. Forty two Honeywell based programs and four DEC-10 programs were created or revised. Of the DEC-10 programs the large molecular energy minimization program GEMO and the data transfer program LOAD are particularly significant. The former augments the programs available in the CHEM group (10 programs) and various CNDO/INDO programs used by laboratory members (see Project No. Z01-AM-29012-07-LCP). LOAD permits standard archive tapes from the Honeywell system to be loaded onto the DEC-10 discs in a format readable from FORTRAN programs or from the data processing utility MLAB.

All documentation for programs, subprograms, operations and device characteristics has been revised and Volume I was distributed in December. Since the operating system will be reconfigured before Jan. 1978, all paper tape source was sorted, categorized and filed in a new cabinet. Honeywell hardware and software manuals have also been sorted and refiled so that basic system documentation is reasonably ordered and accessible should a systems programmer require it.

Significance:

This laboratory computer facility is a integral component in the projects of certain investigators in LMB and LCP. These projects incorporate computer-based data acquisition for a number of reasons but increasingly because of a need to use the sophisticated data processing techniques available on digital computers.

| SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space) | U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT** | PROJECT NUMBER Z01-AM-29016-02-LCP |
|---|---|---|

PERIOD COVERED
July 1, 1976 to September 30, 1977

TITLE OF PROJECT (80 characters or less)
Macromolecular assembly reactions

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT


PI: James Hofrichter — Staff Fellow — A LCP

COLLABORATORS: William A. Eaton — Senior Surgeon — A LCP
Frank Ferrone — Staff Fellow — A LCP


COOPERATING UNITS (if any)

LAB/BRANCH
Laboratory of Chemical Physics

SECTION
Spectroscopy and Structure

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20014

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)


Two major projects have been pursued during the past year: (1) the light scattering photometer described in the 1975/1976 annual report has been improved and used to study the polymerization of hemoglobin S. Control experiments on dilute hemoglobin solutions have also been completed. The kinetic progress curves for the polymerization exhibit a clear delay, but the appearance of significant changes in the scattered intensity can be observed much earlier in the course of the reaction. From these experiments the reaction appears to follow almost a purely autocatalytic time course through the first three decades of detectable signal change; (2) a study of the polymerization of sickle cell hemoglobin partially liganded with carbon monoxide has been completed. This system provides a model for allosteric control of an assembly reaction by bound ligands and is also relevant to sickling in vivo since the polymerizing hemoglobin in red cells is nearly always partially saturated with ligand. Using linear dichroism measurements on the aligned polymer phase both partially and fully liganded hemoglobin molecules were found to be excluded from the polymer phase. The solubility and kinetic behavior of the partially liganded solutions (up to 70%) can be rational-

ized by invoking only the excluded volume of the liganded hemoglobin molecules and very limited copolymerization.

Methods Employed:

Both theoretical and experimental techniques are being used to study assembly reactions. The DC intensity of light scattered by assembling protein solutions is being studies as a function of angle and protein concentration in order to obtain information on the molecular weights and virial coefficients of the proteins and non-assembling controls. The intensity of scattered light is also being used as a probe to examine the kinetics of the polymerization and/or assembly reactions. The immediate goal is to provide a detailed model for the nucleation and growth of the hemoglobin S polymers which is consistent with both the available thermodynamic and kinetic results. Preliminary modelling of mechanisms for the nucleation of polymers have been completed by Dr. William A. Eaton (see accompanying report #Z01-AM-29009-04-LCP). These models are now being examined in terms of their ability to explain both the time dependence of the appearance of polymer and the concentration and temperature dependence of the observed rates over a wide range of conditions.

Further, conventional sedimentation techniques which measure the solubility of the hemoglobin S with respect to the polymer phase and turbidimetric measurements of the rate of appearance of the polymer have been supplemented by linear dichroism measurements in an investigation of the polymerization of partially liganded hemoglobin S solutions. The linear dichroism has the advantage of observing only the hemoglobin which is spontaneously aligned and thus is capable of spectrophotometrically monitoring the composition of the polymer phase without interference from the protein in the solution phase.

Major Findings:

The results to date fall into three major categories: light scattering; solubility and kinetic studies on partially liganded hemoglobin S; and solubility and kinetic studies under new solvent conditions which are designed to help elucidate the polymerization mechanism.

I. Light Scattering

From my previous studies on this system it is clear that there is a well defined delay before the onset of polymerization. This delay is apparent when the reaction is monitored by a variety of independent physical properties, including heat absorption, birefringence, turbidity, viscosity and NMR water linewidths and relaxation times. All of these techniques have limited sensitivity and hence are incapable of providing direct information on the nucleation events which take place during the delay and which determine its length. Presumably the lack of detectable signals is due to the fact that only a small fraction of the sample participates in the formation of nuclei.

Intensity light scattering is proportional to the weight average molecular weight and hence the formation of dimers from all of the molecules in a specific

sample would be expected to increase the scattered intensity by a factor of two. Consequently, I have built a light scattering photometer utilizing a 1.06 micron neodymium laser as a light source in order to monitor the intensity of scattered light in a nearly transparent region of aqueous deoxyhemoglobin solutions.

The instrument allows the resolution of changes in the scattered intensity at 90° to the incident beam which are less than one part in $10^5$ of that scattered by the fully polymerized sample. Light scattering progress curves have been collected on a variety of samples. The progress curve examined with this sensitivity exhibits a shortened delay time, due primarily to the increased sensitivity but, more importantly, the form of the kinetic progress curve can be much more accurately assessed. Instead of exhibiting a time dependence which can be fit by simply raising the time to a high power, approximately 15, as had been previously reported from experiments at lower sensitivity, the intensity scattering, as well as more sensitive turbidity measurements appear to have an exponentially increasing form during the early stages of the reaction. It is also clear that the majority of the light scattered by the fully polymerized sample is scattered into small angles, indicating that the scattering particles must be large compared to the wavelength of light. There is not yet sufficient data on the angular dependence to determine whether the scattering envelope is dependent on a variety of experimental parameters (concentration, temperature, rate of polymerization, time after polymerization) which might be anticipated to alter the shape of the envelope.

Light scattering from dilute hemoglobin solutions at 1.06 μ is complicated by two problems. First, the reciprocal $\lambda^4$ dependence of the scattered intensity results in only marginally measurable excess intensity from dilute hemoglobin solutions (C < .02g/ml). Second, the long wavelength makes the scattering volume large and consequently inter particle scattering begins to contribute at much lower concentrations than at ordinary optical wavelengths. Together these problems have made it difficult to accumulate sufficient reliable data on the scattered intensity to allow an accurate characterization of the monomeric protein.

II. Studies on Gelation of Partially Liganded HbS:

The techniques described above have been used to study stable samples of purified hemoglobin S partially saturated with carbon monoxide. The specific aim of these studies was to answer five important questions regarding the partially saturated system:

1. Does the presence of ligand alter the simple picture of the monomer-polymer equilibrium as a solubility equilibrium between a macroscopic crystal and a solution phase which has been found to apply to deoxyhemoglobin?

2. What is the dependence of the solubility on the fractional saturation of the solution phase with CO?

3. What is the fractional saturation of the polymer phase as a function of the fractional saturation of the solution phase?

4. Can the solubility results obtained from these studies explain the decreased affinity of concentrated hemoglobin S solutions for oxygen?

5. Do the polymerization kinetics in this more complicated system correspond to those observed in the pure deoxyhemoglobin S system?

Sedimentation experiments, in combination with the linear dichroism measurements which show only very small amounts of CO liganded hemes in the polymer, allow clear answers to the first three of these questions, and provide consistent values for the composition of the polymer and solution phases which exist in equilibrium. The partitioning of the ligand between these two phases, a critical test of models for the gelation of multicomponent systems, can also be evaluated directly. The dichroism experiments show that the partition coefficient for the fully liganded $Hb(CO)_4$ molecule must be at least 100. The linear dichroism and solubility studies show that although incompletely liganded hemoglobin molecules, particularly Hb(CO), may be incorporated in the polymer to a significantly greater extent, there is still some selection against these molecules relative to totally deoxygenated HbS.

The solubility results can be used directly to predict a decrease in oxygen affinity ($p_{50}$) with increasing hemoglobin S concentration which is in good agreement with recently reported measurements. This result supports the contention that the change in oxygen affinity arises directly from the preferential polymerization of deoxyhemoglobin S.

The kinetics of the polymerization reaction are very similar to those observed in pure deoxyhemoglobin S solutions, exhibiting delays which are dependent on roughly the 30th power of the supersaturation ratio, that is, the ratio of the total hemoglobin concentration to the solubility. As the fractional saturation is increased from 0% to 40% at constant total hemoglobin concentration, the delay time for gelation increases by about a factor of about 10,000.

Models have been developed in an attempt to account for the solubility results at intermediate degrees of saturation. The essential feature of these models is the application of solution nonideality, as measured by sedimentation and osmotic pressure experiments to the calculation of the deoxyhemoglobin activity in the solution phase. The apparent cooperation of liganded species in the gelation of deoxyhemoglobin, as evidenced by the decreased solubility of deoxyhemoglobin S on the addition of liganded or other hemoglobins to the supernatant arises primarily from their contribution to the solution phase nonideality. Since this effect is totally nonspecific, it could account for the striking similarity in the results of MGC studies on a variety of cyanmethemoglobin species.

III. Kinetic studies under different solvent conditions:

In order to test the predictions of a simple steady state model for the nucleation kinetics, experiments have been carried out under solvent conditions where the solubility of deoxyhemoglobin S is markedly reduced. These include increasing ionic strength and utilizing $D_2O$ instead of water as a solvent.

The theory, together with the analysis of solution nonideality predict that the measured concentration dependence of the reaction should decrease to about 10th order at low concentrations, and that the apparent supersaturation ratio, $c/c_s$, required to achieve a given delay time should increase. The present experiments qualitatively confirm these predictions and will be pursued in order to provide accurate data with which to test this model.

Proposed Course of Project:

The short term goals of the project have been outlined in the methods section and in the progress report. Light scattering from dilute solutions of hemoglobin at 1.06 microns has proven difficult to measure. Consequently these studies will be continued at shorter wavelengths using either conventional illumination or a 632 nm helium-neon laser. A new cell designed to reduce the level of stray light will also be constructed.

The angular dependence of the scattered intensity in gelling solutions will be characterized at predetermined points of the assembly reaction in order to estimate particle sizes at these times. In addition, the angular dependence of the polymerized material at the end of the reaction will be measured as a function of concentration, temperature and delay time.

The highly reproducible progress curves obtained from monitoring the scattering of 90 degrees will also be accumulated as a function of these three variables in order to determine whether or not there is any reproducible change in shape of the curve.

The study of the gelation of partially saturated HbS solutions will be continued by Dr. Frank Ferrone (see accompanying report #Z01-AM-29009-04-LCP) in his laser photolysis experiments on partially liganded solutions. These experiments will generate the necessity for more detailed solubility studies as the experiments progress. The arrival of a new Hemoscan oxygenation instrument will also allow the examination of kinetic progress curves on samples partially liganded with oxygen.

Continued studies in solvent systems which lower the solubility of deoxyhemoglobin S will be given high priority as a means of elucidating the mechanism of the polymerization reaction.

Publications:

Hofrichter, J., Ross, P. D. and Eaton, W. A.: Supersaturation in sickle cell hemoglobin solutions. Proc. Nat. Acad Sci. USA 73: 3035-3039, 1976.

Ross, P. D., Hofrichter, J. and Eaton, W. A.: Thermodynamics of gelation of sickle cell deoxyhemoglobin. J. Mol. Biol., in press.

REPORTED IN PRESS - 1976

Hofrichter, J., Ross, P. D. and Eaton, W. A.: A physical description of

hemoglobin S gelation. In Hercules, J. I., Cottam, G. L., Waterman, M. R. and Schechter, A. N. (Eds.): Proceedings of the Symposium on Molecular and Cellular Aspects of Sickle Cell Disease. DHEW Publication No. (NIH) 76-1007, National Institutes of Health, Bethesda, Md., 1976, pp. 185-223.

ANNUAL REPORT
LABORATORY OF PHYSICAL BIOLOGY
NATIONAL INSTITUTE OF ARTHRITIS, METABOLISM, AND DIGESTIVE DISEASES

The Laboratory of Physical Biology is carrying out studies in a broad range of research areas.

## Muscle Contraction

X-ray diffraction. Instrumentation was developed for examining simplified muscle preparations, under direct chemical control, by X-ray diffraction. Diffraction patterns were recorded from chemically skinned, calcium activated, muscle fibers for the first time. Small ($\sim$ 100 μm in diameter) bundles of rabbit psoas muscle were treated briefly with 20% glycerol - 80% relaxing solution and mounted in a standard relaxing medium. The calcium concentration (pCa) was then varied by changing the bathing solution. The intensity ratio of the first two equatorial reflections, $I_{11}/I_{10}$, increased when the fiber bundle was transferred from relaxing solution to an activating solution at pCa 4.5. When the bundle was returned to relaxing solution, the ratio $I_{11}/I_{10}$ returned to its previous value. Preliminary experiments indicate that the value $I_{11}/I_{10}$ at pCa 4.5 is about 25% higher than at pCa 9. (Lymn, Yu, Podolsky)

Interpretation of the diffraction patterns. Equatorial X-ray reflections from skeletal muscle have been used extensively to study cross-bridge formation during various phases of physiological activity. It has generally been assumed that the ratio of the intensities of the first two equatorial reflections, $I_{10}/I_{11}$, is a measure of the instantaneous number of cross-bridges. This assumption was examined experimentally in frog sartorius muscle by producing different isometric force levels with different concentrations of caffeine and recording the diffraction patterns at the same time. It was found that the intensity ratio $I_{10}/I_{11}$ indeed varies with isometric force, and that the relation is approximately hyperbolic. Variations in the individual peak intensities, $I_{10}$ and $I_{11}$, were also studied as a function of isometric force. $I_{10}$ drops steeply to a steady level when the muscle changes from the resting state to a force level of about half maximal value, whereas $I_{11}$ varies linearly with force. Thus the ratio $I_{10}/I_{11}$ is a sensitive indicator of cross-bridge number at low isometric force (< 50% of maximal force), whereas its inverse is more sensitive at high force levels. (Hartt, Yu, Podolsky)

Further calculations using a three-dimensional form factor showed that the redistribution of myosin subfragment-1 moieties resulting from their attachment to actin filaments can, by itself, explain the characteristic change in equatorial X-ray reflections when vertebrate skeletal muscle is activated. A change in the configuration of an existing actin-subfragment-1 complex can also have a profound effect on the equatorial pattern. For

a given configuration of actin-subfragment-1 complex, the reflection intensities are probably a non-linear function of attachment number, although a plot of intensity ratio against attachment number may approximate a straight line when the attachment number is small. (Lymn)

A two dimensional X-ray detector. Using techniques learned in the construction of linear position-sensitive proportional counters, a new type of X-ray detector for use in the study of muscle function was designed, built and tested. This new detector makes it possible to observe equatorial and meridional X-ray reflections simultaneously without much more sophisticated electronics than that required for a one-dimensional detector. (Sand, Crist)

Cross-bridge rate functions. A new technique was developed in this laboratory for determining muscle stiffness by measuring the propagation speed of small mechanical disturbances. Recently, a supplemental technique was worked out for measuring muscle sarcomere movements using laser diffraction methods. This was necessary because sarcomere motion can influence both muscle force and stiffness. Combined measurements of force, stiffness, and sarcomere motion have suggested that motion does not cause the number of cross-bridges to fall in the way required by A. F. Huxley's 1957 rate constants. This inference was drawn from the calculated effect of sarcomere shortening upon force and stiffness. A more direct approach is to measure force and stiffness following activation under conditions in which sarcomere motion is totally suppressed with a feedback method, and efforts are currently underway in this direction. It is interesting to note that these findings are consistent with recent X-ray data from this Laboratory which show that the equatorial diffraction pattern from frog muscle is insensitive to motion. (Schoenberg)

Ionic strength and the force-velocity relation. Mammalian muscle cell membranes can be made permeable to physiological solutions by treatment with glycerol. When rabbit psoas fibers immersed in 50% glycerol are first rinsed with a 20% glycerol-salt solution before being transfered to a rigor or relaxation solution, the mechanical properties were found to be reproducible to the same extent as with frog skinned fibers. Ionic strength changes the curvature of the force-velocity relation of rabbit fibers, without affecting the maximum shortening velocity. (Tawada) This contrasts with the behavior of mechanically skinned frog fibers, where the steady motion is unaffected by ionic strength. Ionic strength also changes the isotonic velocity transients, which shows that this parameter modifies the cross-bridge rate functions. (Gulati)

The activation mechanism. Intracellular calcium movements in isolated skinned muscle fibers were examined with isometric force measurements and improved tracer methods. Further studies of "depolarization"-induced calcium release in the presence of $10^{-4}$M magnesium ion show that a) stimulation of $^{45}Ca$ efflux is undetectable when calcium in the myofilament space is chelated, suggesting that the response has an absolute calcium requirement; b) stimulation of

$^{45}Ca$ release is inhibited by stretch to twice the normal sarcomere length, suggesting that structural integrity of some region of the internal membrane systems is necessary for coupling depolarization to calcium release in the skinned fiber; c) stimulation of net calcium release is inhibited when bath potassium is substituted by choline or tris ions, suggesting that potassium movement across the internal membranes may be an important ionic mechanism underlying calcium release. (Stephenson)

## Cell Membranes

Lipid complexes. The formation of a lecithin:cholesterol 2:1 complex in water has been shown to be responsible for two phenomena that in the past have been poorly understood, but which have had an important influence on the current ideas of the effect of cholesterol on membrane structure and function. The first is the "condensing" effect of cholesterol on lecithin films, and the second is the decrease in non-electrolyte permeability upon addition of cholesterol to multilamellar lecithin bilayers in water dispersions. In each case the presence of the 2:1 complex has been able to account quantitatively for the phenomenon. In general the presence of cholesterol and phospholipids at compositions other than the 2:1 ratio of the complex will result in the formation of two phases: complex and the pure phase of the other component which is in excess. Therefore, cell membranes whose cholesterol content is varied will have their lipids in a heterogeneous state.

The phospholipids of cell membranes are presently considered to be arranged in a single bilayer which can be thermally induced to undergo a crystal-liquid crystal phase transition. It has been inferred from this that a portion of the membrane lipids can exist in a crystalline state. However, analysis of the internal energies of phospholipids in a variety of bilayer states indicates that the hydrocarbon moieties are invariably liquid-like under conditions which are typical for biological systems; the only crystalline phospholipid state which has been observed is multibilayer. Therefore reports of crystalline arrays of membrane lipids suggests that a portion of the lipids in membranes is in a multibilayer state. (Gershfeld)

Red cell membrane. Studies were continued of the significance of the limit to the extractability of erythrocyte membrane cholesterol by plasma lipoproteins. Neither the kinetics of the extraction, nor the maximum amount extractable, are a function of the ages of the cells. Despite the fact that only a fraction of the membrane cholesterol is extractable, all of it is exchangeable for (labelled) cholesterol incorporated into lipoproteins. The kinetics of re-extraction of cholesterol, from cells in which the initially extractable cholesterol was replaced by labelled cholesterol, confirm the presence of two cholesterol pools and indicate a slow exchange between them. To follow up on these observations, work was started on the measurement of fluorescence

resonance energy transfer between membrane proteins and certain polyene antibiotics which form fluorescent complexes with cholesterol, since this type of measurement provides additional information about membrane structural changes involving cholesterol. (Gottlieb)

## Morphogenesis

Measurement of cellular DNA. Measurement of DNA in Euglena gracilis by flow microfluorometry was studied previously. Although this method is rapid and sensitive, it only gives relative values. In collaboration with Dr. Vincent Bono, NCI, measurements of cellular DNA were made in the protozoan, Euglena gracilis, and in mouse leukemia lymphoblasts. Both microfluorometric determinations of the DNA distribution in normal cell populations and the average cellular DNA measured by classical chemical determinations (diphenylamine and indole reactions) were obtained. Similar measurements made on cultures exposed to conditions that alter cellular DNA levels (colcemid and ethanol for mouse cells, vitamin $B_{12}$ starvation for Euglena) showed that the quantitative change in DNA per cell measured by the two techniques was the same. For the first time, the DNA distributions among entire populations of protozoan and mammalian cells were established on an absolute scale. (Kempner)

Pineal gland. Post-natal development of the rat pineal gland was studied in collaboration with Drs. D. Klein and M. Buda, NICHHD. At birth, isolated glands contain no hydroxy indole-O-methyl transferase activity, an enzyme in the serotonin pathway which ultimately leads to melatonin. The missing enzyme activity was measurable by age five days, and continued to increase linearly for 3 weeks. Trypsinization of the tissue at birth releases a homogeneous population of large cells (308 $\mu m^3$ volume) with large nuclei. Cell volume increases continuously for three weeks, increasing about 50% on the average. Between 23 and 32 days there is an appearance of small cells (52.7 $\mu m^3$) which increase in number but not volume, while the proportion of large cells drops to zero. By age 35 days, only the small cells are seen and these persist throughout life. (Kempner)

Insect metamorphosis. Insect metamorphosis is a programmed process consisting of three phases: (1) an actively growing and feeding stage--the larva--the primary purpose of which is the accumulation of sufficient energy reserves for the subsequent metamorphosis; (2) pupation and adult development wherein most of the larval cells and organs are degraded to provide the "raw materials" for the adult organs; (3) adult maturation and reproduction by the winged stage for distribution of the species. Calliphorin is the major storage protein of the blue blowfly; analogous proteins are found in several other insects. The concentration of calliphorin in the blood increases with the duration of larval feeding; at the cessation of feeding, during the so-called "wandering stage," calliphorin synthesis by the fat body is abruptly turned off, and the blood calliphorin is re-adsorbed by the fat body in the form of insoluble, intracellular granules. The eventual fate of these granules

is still unknown. In fat body cultivated in vitro the amount of protein, including calliphorin, synthesized and excreted into the medium depends on the amount and composition of the medium, the components of the gas phase, the age of the larva, and the concentration of molting hormone (β-ecdysone). For the most part, β-ecdysone at physiological concentrations inhibits protein synthesis in general, and calliphorin synthesis in particular. This effect probably reflects a natural function of the molting hormone in metamorphosis. (Pau, Levenbook)

Human red cell sickling. Sickle cell disease is a non-infectious hereditary anemia in which the disease process is well understood at the molecular level. The defective molecule has not only been identified as an abnormal hemoglobin (Hb S), but the abnormal interactions of this molecule can be explained. Observations reported from many laboratories are consistent with an aggregation mechanism due to hydrophobic interactions by deoxy Hb S, as postulated by Murayama. Studies with the inorganic ions $Cl^-$, $Br^-$, $I^-$, and $SCN^-$ show that both aggregation and solubility of deoxy Hb S are inhibited by these ions in the order of the classical Hofmeister series. It was also found that red cells increase in volume upon "sickling." This change is in the direction of the volume increase during sickling observed by Murayama in high hydrostatic pressure experiments and is consistent with his thermodynamic prediction of the volume of activation. In collaboration with Drs. Oku Ampofo, Roger Lewis, J. Obeng, and M. Appiah in Ghana, additional data have been obtained concerning the effectiveness of oral carbamide in both the prevention and amelioration of sickle cell crisis. (Murayama)

## Bioluminescence

Literature reports of bioluminescent phenomena in a wide variety of animals, mostly marine, have been reviewed. The data indicate that light emission has been adapted during evolution to at least twenty diverse functions. Among the most surprising adaptations are the employment of symbiotic luminous bacteria in light organs of certain squids and fishes, the use of ventral body illumination in deepwater squids and fishes to obliterate their daytime silhouettes as seen by predators swimming below, and the coded courtship flashing systems of many firefly species. The evidence indicates that (1) bioluminescence involves several chemically diverse and independently evolved light-emitting systems, (2) luminescence followed, not preceded, the adoption of nocturnal habits or dark habitats, (3) the evolution of adaptations such as courtship signals or recognition via emission color - that is, uses of bioluminescence that depend on lock and key or mirror-image genetic fixation of mutations in different individuals (e.g., male and female) - seem to require a "holding" status for each of the progressive steps in the evolution of the adaptation, in line with the indications of neutral mutations and gene copying in procaryotes. (Buck)

| SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space) | U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT | PROJECT NUMBER Z01 AM 27000-15 LPB |
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PERIOD COVERED
July 1, 1976 through September 30, 1977

TITLE OF PROJECT (80 characters or less)
The Mechanism of Muscular Contraction

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

| | | | |
|---|---|---|---|
| PI: | R. J. Podolsky | Chief, LPB | LPB NIAMDD |
| OTHER: | R. W. Lymn | Senior Staff Fellow | LPB NIAMDD |
| | L. C. Yu | Senior Staff Fellow | LPB NIAMDD |
| | E. W. Stephenson | Guest Worker | LPB NIAMDD |
| | J. Gulati | Staff Fellow | LPB NIAMDD |
| | J. E. Hartt | Staff Fellow | LPB NIAMDD |
| | K. Tawada | Guest Worker | LPB NIAMDD |

COOPERATING UNITS (if any)
None

LAB/BRANCH
Laboratory of Physical Biology

SECTION
Section on Cellular Physics

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20014

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Muscle cells are activated when calcium is released from the sarcoplasmic reticulum into the myofilament space. The processes involved in this intracellular calcium movement have been studied by measuring isometric force and $^{45}Ca$ fluxes in mechanically skinned fibers from frog muscle. The structural changes associated with the transition of muscle cells from one physiological state to another have been examined by X-ray diffraction techniques, using radiation from conventional rotating anode generators and synchrotrons in conjunction with electronic, position sensitive, X-ray detectors. The rate constants associated with the turnover of actomyosin cross-bridges have been studied by analyzing the contraction kinetics of skinned muscle fibers under controlled chemical conditions.



PHS-6040
(Rev. 10-76)

Project Description:

Objectives:

1. To work out the molecular mechanism of muscular contraction.
2. To understand the control processes for contractility.

Methods Employed:

1. Analysis of the motion and the X-ray diffraction pattern of both intact muscle fibers and "skinned" fiber segments under chemically controlled conditions.
2. Study of the electrical and chemical parameters involved in the activation of various types of muscle fibers.
3. Measurement of ionic fluxes across the internal membranes of skinned muscle fibers under various conditions.

Major Findings:

1. The equatorial X-ray reflections of frog skeletal muscle were measured as a function of the isometric force level, which was controlled by caffeine. The intensity ratio $I_{11}/I_{10}$ is proportional to force, which shows that increments in contractile force are caused by increments in cross-bridge number. Variations in the individual peak intensities, $I_{10}$ and $I_{11}$, were also examined. $I_{10}$ dropped steeply to a steady level when force increased from the resting value to about half the maximum value, while $I_{11}$ increased linearly with force. Therefore $I_{10}/I_{11}$ is a sensitive indicator of cross-bridge number at low isometric force levels, while $I_{11}/I_{10}$ is more sensitive at high force levels.

2. X-ray reflections were obtained from chemically skinned, calcium activated, rabbit psoas fibers. The intensity ratio $I_{11}/I_{10}$ at pCa 4.5, when full isometric force was developed, is about 25% higher than in relaxed fibers at pCa 9.

3. Calculations using a three dimensional form factor show that a change in structure of an existing actin-subfragment-1 complex can have a profound effect on the equatorial diffraction pattern. For a given configuration of the complex, the reflection intensities may be a non-linear function of attachment number, though a plot of intensity ratio, $I_{11}/I_{10}$, may approximate a straight line when attachment numbers are small.

4. A new electronic position-sensitive detector, which records both equatorial and meridional X-ray reflections, was designed, constructed. and tested.

5. Intracellular calcium movements in isolated skinned muscle fibers were examined with isometric force measurements and improved tracer methods. Studies of "depolarization"-induced calcium release in the presence of $10^{-4}$M magnesium ion indicate that a) the response has an absolute calcium requirement; b) structural integrity of the internal membrane systems is necessary for coupling depolarization to calcium release; c) potassium movement across the internal membranes may be an important ionic mechanism underlying calcium release.

6. The contraction kinetics of extensively glycerinated rabbit psoas fibers were studied as a function of ionic strength. The curvature of the relative force-velocity relation is sensitive to ionic strength, but the maximum shortening velocity is unaffected. This contrasts with the steady motion of mechanically skinned frog muscle fibers, which is unaffected by ionic strength.

Significance to Biomedical Research and the Program of the Institute:

The elucidation of the molecular mechanism of muscular contraction together with the chemistry of the activation process, can be useful in the rational handling of neuromuscular and cardiovascular disease.

Proposed Course of Project:

1. The influence of various agents (pCa, pH, ionic strength, metal ions, ATP) on the ability of muscle fibers to develop force and to shorten will be examined directly in skinned fiber segments.
2. The influence of these same agents on myofilament spacing and the cross-bridge configuration will be examined in skinned fibers by X-ray diffraction.
3. The effect of motion on the number of cross-bridges in intact, electrically-stimulated muscle fibers will be studied by X-ray diffraction.
4. Movement of calcium and magnesium between the myofilament space and the sarcoplasmic reticulum will be studied by tracer methods.

Publications:

Stephenson, E. W. and Podolsky, R. J.: Regulation by magnesium of intracellular calcium movement in skinned muscle fibers. J. Gen. Physiol. 69: 1-16, 1977.

Stephenson, E. W. and Podolsky, R. J.: The influence of magnesium on chloride-induced calcium release in skinned muscle fibers. J. Gen. Physiol. 69: 17-35, 1977.

Podolsky, R. J. and Stephenson, E. W.: The function of the internal membrane system in muscle cells. Proc. of the MDA 5th Int. Sci. Conf. In press.

Gulati, J.: Force-velocity characteristics for calcium-activated mammalian slow-twitch and fast-twitch skeletal fibers from the guinea pig. Proc. Natl. Acad. Sci. USA 73: 4693-4697, 1976.

Palmer, L. C. and Gulati, J.: Potassium accumulation in muscle: A test of the binding hypothesis. Science 194: 521-523, 1976.

Yu, L. C., Lymn, R. W. and Podolsky, R. J.: Characterization of a non-indexible equatorial X-ray reflection from frog sartorius muscle. J. Mol. Biol., In press.

| SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space) | U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT** | PROJECT NUMBER Z01 AM 27001-03 LPB |
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PERIOD COVERED
July 1, 1976 through September 30, 1977

TITLE OF PROJECT (80 characters or less)
Contractility and Excitability of Skeletal and Cardiac Muscle

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: Mark Schoenberg Medical Officer LPB NIAMDD

COOPERATING UNITS (if any)
Dr. Jay B. Wells, Medical Neurology Branch, NINCDS

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SUMMARY OF WORK (200 words or less - underline keywords)

Our study to determine the mechanism of muscle contraction will concentrate upon separating sarcomere motion from motion at the ends of the muscle, the former being greatly influenced by tendon compliance. In studying this we will make use of optical diffraction techniques. Feedback will be used to control sarcomere motion. Stiffness measurements based upon the speed of propagation of small mechanical disturbances, will be used as a measure of cross-bridge interaction during isotonic shortening.



PHS-6040
(Rev. 10-76)

Project Description:

Goal: To understand the molecular and structural basis of muscular contraction.

Methods and Major Findings:

1) Over previous years, we have developed a technique for measuring muscle stiffness by measuring the propagation speed of small mechanical disturbances. This has enabled us to follow the development of force and stiffness during a twitch. Both of these are influenced by the interaction of the muscle sarcomeres with the muscle tendon. It is largely the properties of the muscle sarcomeres which are of importance in getting at an understanding of the molecular basis of contraction.

2) In the previous year, we have refined a laser diffraction light position sensing device for monitoring the movement of muscle sarcomeres during contraction. Although our multifiber preparation shows a fair amount of nonuniformity, we have found that in many regions the sarcomeres shorten as if a compliance of up to 0.08 $L/P_o$ is in series.

3) The series compliance will delay the appearance of force relative to "activation." The stiffness will also be delayed, the extent of this delay depending upon the rate constant for the formation of bridges. By measuring the size of the compliance (Finding 2) and the delay between stiffness and force (Finding 3, FY 1976), we have been able to place restrictions on the rate constant for making bridges. The rate constants are such that number of cross-bridges in a contracting muscle would seem to be somewhat greater than predicted by the original rate constants suggested by A. F. Huxley (Prog. Biophys. 7:255, 1957).

Significance to Biomedical Research and the Program of the Institute:

Experiments on the mechanism of muscular contraction are significant for two reasons. First they contribute to a sound understanding of how normal muscle works (which is vital to any study of diseased muscle) and secondly, the same mechanisms responsible for muscle contraction appear to be common to many other cells in which motility plays a role such as platelets, nerve axons, and many others.

Future Course:

A more direct way of learning about the cross-bridge rate constants is to prevent totally any sarcomere shortening during activation. To date, we have used our laser diffraction light position sensor to record the sarcomere

shortening that normally exists. We need to use this signal as a feedback signal so that we can prevent any sarcomere shortening by compensatorily pulling on the ends of the muscle with a motor. We have been prevented from doing this by the non-uniformity of our multifiber preparations. In the coming year we hope to develop techniques for performing these measurements on the more uniform single fiber preparation.

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PERIOD COVERED July 1, 1976 through September 30, 1977

TITLE OF PROJECT (80 characters or less)

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NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: John B. Buck Research Physiologist LPB NIAMDD

COOPERATING UNITS (if any) Mrs. Elisabeth M. Buck, Guest Worker; Dr. Frank A. Hanson, University of Maryland, UMBC, Catonsville, Maryland; Dr. James F. Case, University of California, Santa Barbara, California.

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SUMMARY OF WORK (200 words or less - underline keywords)

The imminent completion of the series of long-gestation papers on flash pacemaking in New Guinea fireflies was again interrupted by the principal investigator's inability to resist an invitation to write an overview chapter for a symposial monograph on bioluminescence. The choice of subject -- function and evolution of bioluminescence -- was very educational because it required critical review and rethinking of a large corpus of literature, primarily on marine animals, and an attempt to view the question of the origin and development of the polyphyletic ability to emit light in the light of modern concepts of molecular biology. With regard to the pacemaker work, Dr. Hanson completed a computerized retrieval program by means of which we processed several thousand feet of tape-recorded data that had previously either not been analysed at all or only in fragments. Mrs. Buck has analysed most of the field data (two and three channel synchrony) among these records.

Project Description:

Objectives:

1. We hope to produce a unified description and theory of the five types of firefly flash synchronization thus far studied that will define the central nervous control pacemaker. One promising recent lead is Dr. Hanson's success in relating some of the data to circadian data ascribed to weak and strong "Zeitgebers."

2. It is possible that we can have some influence in bringing about better rapprochement between the fields of molecular genetics and comparative physiology, fields thus far almost completely independent.

Significance to NIAMDD Research:

Insofar as the Institute supports attempts to broaden our understanding of basic biological processes it is hoped that our work is appropriate.

Proposed Course of Project:

No major change anticipated.

Publications:

Buck, J. and Buck, E.: Toward a functional interpretation of synchronous flashing by fireflies. Am. Nat. (In Press).

REPORTED IN PRESS - 1976

Hanna, C. H., Hopkins, T. A. and Buck, J.: Peroxisomes of the firefly lantern. J. Ultrastructure Res. 57: 150-162, 1976.

| SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space) | U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT** | PROJECT NUMBER Z01 AM 27003-08 LPB |
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PERIOD COVERED
July 1, 1976 through September 30, 1977

TITLE OF PROJECT (80 characters or less)
The Dynamic Properties of Cell Membranes and Related Systems

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

| | | | |
|---|---|---|---|
| PI: | Norman L. Gershfeld | Research Chemist | LPB NIAMDD |
| OTHER: | Kazuo Tajima | Guest Worker | LPB NIAMDD |

COOPERATING UNITS (if any)
S. Razin, Department of Clinical Microbiology, Hadassah Medical School Jerusalem, Israel

LAB/BRANCH
Laboratory of Physical Biology

SECTION
Section on Comparative Physiology

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20014

| TOTAL MANYEARS: | PROFESSIONAL: | OTHER: |
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| 2.3 | 1.3 | 1 |

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The formation of lecithin:cholesterol 2:1 complexes in water has been shown to be responsible for two phenomena that in the past have been poorly understood, but which have had an important influence on the current ideas of the effect of cholesterol on membrane structure and function. The first is the "condensing" effect of cholesterol on lecithin films, and the second is the decrease in non-electrolyte permeability upon addition of cholesterol to multilamellar lecithin bilayers in water dispersions. In each case the presence of 2:1 complex has been able to account quantitatively for the phenomenon. In general the presence of cholesterol and phospholipids at compositions other than the 2:1 ratio of the complex will result in the formation of two phases: complex and the pure phase of the other component which is in excess. Cell membranes whose cholesterol content is varied will therefore have their lipids in a heterogeneous state. Analysis of the internal energies of phospholipids in a variety of thin film states of aggregation indicates that the hydrocarbon moieties are liquid-like under conditions which are typical for biological systems.

Project Description:

Major Findings:

I. Physical-chemical properties of membrane lipids.

A. Manifestations of lecithin:cholesterol 2:1 complex formation.

The formation of lecithin:cholesterol 2:1 complexes in water (reported in 1976) has now been shown to occur in surface as well as bulk states. Coexistence of surface phases of 2:1 complex and either lecithin or cholesterol (depending on the mole fraction of cholesterol X(CHOL) present) has been demonstrated. The implications of these results have been examined in two systems. The first is the spread monolayer system of lecithin and cholesterol in which a "condensing" effect of cholesterol on lecithin has been reported by many investigators since 1925, but no quantitative description of the phenomenon has been presented until now. The concept that lecithin and cholesterol formed homogeneous surface solutions has led to ad hoc rationalizations of the experimental data. Some of the lecithins do not exhibit the "condensing" effect with cholesterol, and the effect is not predictable based on the chemical nature of the lecithin. However this phenomenon has been resolved by the presence of 2:1 complex in the surface film; complex formation is a general property of all lecithins (and for other phospholipids), and all the surface film data can be accommodated by the coexistence of the 2:1 complex with either lecithin or cholesterol.

The second system is the multilamellar lecithin liposome in water in which the permeability of non-ionic solutes through this particle has been examined as a function of the composition of cholesterol in the liposome. It has been found that the rate of diffusion through the lipid particles decreases as the concentration of cholesterol increases. Interpretation of this phenomenon with the assumption that the components were homogeneously distributed did not adequately describe the phenomenon. However, a quantitative description of the decrease in permeability is obtained if the system is treated as a two phase system: when $0 < X(CHOL) < 0.33$, 2:1 complex coexists with pure lecithin; and when $0.33 < X(CHOL) < 1.0$, 2:1 complex coexists with pure cholesterol phase. If the rates of permeation R of each of these phases is known, the dependence of permeation rates on cholesterol composition can be predicted from the relation

$$R = X_i R_i + X_j R_j$$

where X is the mole fraction of each component in the liposome, 2:1 complex, and lecithin or cholesterol. It has been shown that the entire literature of non-ionic solute permeability for these systems can be treated by this relation.

B. Physical states of membrane lipids.

Reports of phase transitions in intact plasma membranes have indicated that a gel-liquid crystal transition occurs. The implication of this result is that a discrete bilayer (the membrane) can exist with at least a portion of its lipids in a crystalline state. A test of the consistency of this result with the general physical properties of membrane lipids has been performed. Discrete monolayer and bilayer states of the phospholipids have been observed and the internal energies of these systems measured. The results of this analysis indicate that when condensed monolayer and bilayer states are formed by spontaneous processes, the lipids are in liquid-like states. The only crystalline states of the phospholipids which have been observed are the multilamellar states. The implication of this result is that the gel state reported for cell membrane suspensions may represent a multilamellar array as a small portion of the lipids in the membranes.

Future Course:

The dynamics of discrete bilayer formation will be studied as a function of lipid composition.

Publications:

Horn, L. W. and Gershfeld, N. L.: Equilibrium and metastable states of lecithin. Biophys. J. 18:301-310, 1977.

Gershfeld, N. L. and Tajima, K.: Energetics of the transition between lecithin monolayers and bilayers. J. Colloid Interface Sci. 59:597-604, 1977.

Reported in Press, 1976

Gershfeld, N. L.: Physical chemistry of lipid films at fluid interfaces. Ann. Rev. Phys. Chem. 27:349-368, 1976.

| SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space) | U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT** | PROJECT NUMBER Z01 AM 27004-07 LPB |
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PERIOD COVERED
July 1, 1976 through September 30, 1977

TITLE OF PROJECT (80 characters or less)
Physical Chemistry

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

| | | | |
|---|---|---|---|
| PI: | Melvin H. Gottlieb | Research Chemist | LPB NIAMDD |
| OTHER: | Reiji Aoshima | Visiting Fellow | LPB NIAMDD |

COOPERATING UNITS (if any)

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Laboratory of Physical Biology

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Section on Comparative Physiology

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20014

| TOTAL MANYEARS: | PROFESSIONAL: | OTHER: |
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CHECK APPROPRIATE BOX(ES)

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Studies were continued of the significance of the limit to the extractability of erythrocyte membrane cholesterol by plasma lipoproteins. Neither the kinetics of the extraction, nor the maximum amount extractable, are a function of the ages of the cells. Despite the fact that only a portion of the membrane cholesterol is extractable, all of it is exchangeable for (labelled) cholesterol incorporated into lipoproteins. The kinetics of re-extraction of cholesterol, from cells in which the initially extractable cholesterol was replaced by labelled cholesterol, confirm the presence of two cholesterol pools and indicate a slow exchange between them. Work was started on the measurement of fluorescence resonance energy transfer between membrane proteins and certain polyene antibiotics, which form fluorescent complexes with cholesterol. This type of measurement would provide a means of determining membrane structural changes involving cholesterol.



PHS-6040

Project Description:

As in the past few years our work concerned the state of cholesterol in (human) erythrocyte membranes. Previous studies, on the extraction of cholesterol from the membranes by cholesterol-depleted plasma lipoproteins, had indicated that cholesterol exists in "extractable" and "bound" states in the membrane. Since ordinary erythrocyte populations consist of cells of a range of ages, with age-related differences in membrane composition, the early work of this year examined the possibility that the two binding states are related to cell age. Both the kinetics of the cholesterol extraction by lipoprotein, and the maximum amounts extractable, were found to be independent of cell age.

Later work concerned the relationship between the exchange of cholesterol between erythrocyte membranes and lipoproteins, and the extraction process. Studies with lipoproteins into which labelled cholesterol was incorporated showed that all of the erythrocyte cholesterol is exchangeable, even though only 30% is extractable. Preliminary results, on the re-extraction of cholesterol from erythrocytes in which the initially extractable material has been replaced by labelled cholesterol, confirm the existence of two cholesterol pools in the membrane and indicate a slow exchange between the pools.

In collaboration with Visiting Fellow R. Aoshima, work was begun on measurement of fluorescence resonance energy transfer (r.e.t.) between erythrocyte membrane proteins and certain polyene antibiotics, which form fluorescent complexes with cholesterol. Measurement of r.e.t. would provide a means of determining membrane structural changes involving cholesterol, since this quantity is proportional to the distance between the groups involved. Initial studies were done with pimaricin, the least hydrophobic of this series of polyenes, because its slight water solubility permits its introduction into the membrane without using organic solvents. However, it has been concluded that the optical absorbance, fluorescence and membrane-water partition parameters of pimaricin would give r.e.t. values which at best would be only marginally measurable with instrumentation available to us. Studies are currently being carried out with a more hydrophobic polyene, lucensomysin, which, because of a more favorable membrane-water partition coefficient, should give larger r.e.t. values.

Publications:

Gottlieb, M. H.: The reactivity of human erythrocyte membrane cholesterol with a cholesterol oxidase. Biochim. Biophys. Acta 466: 422-428, 1977.

| SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space) | U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT** | PROJECT NUMBER Z01 AM-27006-20 LPB |
|---|---|---|

PERIOD COVERED July 1, 1976 through September 30, 1977

TITLE OF PROJECT (80 characters or less)

Developmental biochemistry of insect metamorphosis

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

| | | | |
|---|---|---|---|
| PI: | L. Levenbook | Research Chemist | LPB NIAMDD |
| OTHER: | A. Bauer | Research Physiologist | LPB NIAMDD |
| | A. Protzel | Visiting Fellow | LPB NIAMDD |
| | R. Pau | Visiting Scientist | LPB NIAMDD |

COOPERATING UNITS (if any)

None

LAB/BRANCH

Laboratory of Physical Biology

St

Comparative Physiology Section

INSTITUTE AND LOCATION

NIAMDD, NIH, Bethesda, Maryland

| TOTAL MANYEARS: | PROFESSIONAL: | OTHER: |
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☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

(1) Some of the reasons why a successful procedure for the routine preparation of pure blowfly calliphorin has not yet been achieved have become apparent. This protein is intrinsically unstable, and spontaneously breaks down into lower Mol. Wt. components. (2) The earlier qualitative observations that molting hormone (β-ecdysone) inhibits fat body synthesis of calliphorin in vitro has been investigated quantitatively. (3) The kinetics of phagocytosis by larval blood hematocytes in vitro has been studied employing a new microassay. These cells contain a fraction of the blood calliphorin. (4) The changing titers of several free monosaccharides during embryogenesis of the tick Dermacentor andersoni have been measured.

Project No. 701 AM-27006-20

Project Description:

Objectives:

1. Purification of the blowfly protein calliphorin as a prerequisite to a study of its structure and function.

2. Hormonal control of calliphorin-biosynthesis *in vitro*.

3. *In vitro* phagocytosis by larval blowfly blood cells.

4. Changes in free sugar titers during embryogenesis of the tick *Dermacentor andersoni*.

Methods Employed:

Routine physical, chemical and immunological procedures; *in vitro* cell and organ culture.

Major Findings:

Calliphorin is intrinsically less stable than most proteins; its breakdown is not haphazard since the breakdown pattern is fairly consistent. A conspicuous example of such instability is the finding that heating with sodium dodecyl sulfate produces a spectrum of low molecular weight polypeptides smaller than the known sub-units. Our purest preparations are water insoluble.

For a more accurate evaluation of larval calliphorin titers in blood and tissues, and changes with age, the technique of "rocket" immunoassay has been employed. However, the standard procedures had to be modified for use at pH's less than 7. The desired "rockets" were achieved by employing suitably carbamylated, purified IgG at pH 6.8. Fat body granules containing calliphorin require SDS for solubilization, and hence SDS-protein -- carbamylated IgG systems for quantitative "rockets" are being tested.

Earlier qualitative observations that molting hormone (= β-ecdysone) inhibits calliphorin synthesis by isolated fat body *in vitro* have been extended to include the effects of a number of variables (e.g. addition of serum albumen to the medium, age of donor larvae, methionine concentration, etc.) in order to place the earlier finding on a firm quantitative footing.

An improved medium and a new micro-assay for phagocytosis by larval blood hematocytes has been employed to follow the time course of such activity under a variety of experimental conditions. Unfortunately, the data are clouded by the inability to distinguish between truly ingested and non-specifically adsorbed particles.

Major Findings: (continued)

The titers of the four principal free sugars of tick eggs and embryos have been measured during Dermacentor andersoni embryogenesis. Major changes in glucose and mannose concentrations were observed.

Significance to NIAMDD Research:

Insect metamorphosis is a programmed process under hormonal control which entails the decay and destruction of the larval cells concomitantly with the differentiation of the cells and structures that will give rise to the adult insect. The greater part of this process has taken place within the span of a week. This telescoping of the aging and development process in the insect might be expected to bring forth features otherwise unobservable in the corresponding processes of man and mammals, which take place in a much more expanded time scale. In particular, the study of the fate of the principal soluble larval protein refers to the fate of an essential component of the developmental machinery and its products during aging and development.

Proposed Course of Projects:

Continuation of studies on structure and function of both calliphorin and of storage granules.

Publications:

Protzel, A., Sridhara, S. and Levenbook, L.: Ribosomal replacement and degradation during metamorphosis of the blowfly, Calliphora vicina. Insect Biochem. 6: 571-578, 1976.

Protzel, A. and Levenbook, L.: Rate of rRNA synthesis during metamorphosis of the blowfly, Calliphora vicina. Insect Biochem. 6: 631-635, 1976.

Chen, P. S., Fales, H. M., Levenbook, L., Sokolski, E. A. and Yeh, H.J.C.: Isolation and characterization of a unique galactoside from male Drosophila melanogaster. Biochemistry (In Press).

Levenbook, L.: Analytical Biochemistry of Insects -- a book review for Pesticide Biochem. Physiol. (In Press).

| SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space) | U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT** | PROJECT NUMBER Z01 AM-27007-15 LPB |
|---|---|---|

PERIOD COVERED July 1, 1976 through September 30, 1977

TITLE OF PROJECT (80 characters or less)

Human red cell "sickling" and "desickling"

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

| | | | |
|---|---|---|---|
| PI: | M. Murayama | Research Chemist | LPB NIAMDD |
| OTHER: | A. Levine | Staff Fellow | LPB NIAMDD |

COOPERATING UNITS (if any)

Dr. O. Ampofo, Dr. Roger Lewis, Dr. J. Obeng, and Dr. M. Appiah, Tetteh Quarsh Memorial Hospital and Ministry of Health, Mampong-Akwapim, Ghana, West Africa. Dr. T. Shehata, LPB, NIAMDD.

LAB/BRANCH

Laboratory of Physical Biology

Comparative Physiology Section

INSTITUTE AND LOCATION

NIAMDD, NIH, Bethesda, Maryland

| TOTAL MANYEARS: | PROFESSIONAL: | OTHER: |
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| 1-1/3 | 1-1/3 | 0 |

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☒ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☐ (c) NEITHER

☒ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)


Human red cell "sickling" and "desickling."

Sickle cell disease is a non-infectious hereditary anemia in which the disease process is well understood at the molecular level: the defective molecule has not only been identified as an abnormal hemoglobin (Hb S), but the abnormal interactions of this molecule can be explained. Observations reported from many laboratories are consistent with an aggregation mechanism due to hydrophobic interactions by deoxy Hb S, as postulated by Murayama. More recently, we have found in our laboratory that both aggregation and solubility of deoxy Hb S are inhibited by inorganic ions in the order of the classical Hofmeister series. We also found that red cells increase in volume upon "sickling." This change opposite in direction to published claims or predictions, is in the direction of the volume increase during sickling observed by Murayama in his high hydrostatic pressure experiment and with his thermodynamic prediction of the volume of activation. In collaboration with Dr. Oku Ampofo, Dr. Roger Lewis, Dr. J. Obeng, and Dr. M. Appiah in Ghana additional data are obtained supporting our previous finding of the effectiveness of oral carbamide in prevention of crisis and ameliorating the symptoms of sickle cell crises.

Project Description:

Objectives:

Essentially the same as last year; however, with the following addition:

To investigate a co-factor of S Hb aggregation in blood platelettes and also excretion of same in patients' urine.

Methods Employed:

Methods are essentially the same as last year; however, the change in density of deoxy S Hb has been investigated by pycnometric methods (density gradient and erythrocyte sedimentation rates) and also the change of density is reflected in its index of refraction, which is measured with Bausch and dipping refractometer. Because it is assumed that electrostriction occurrs when polymerized, S Hb is transformed to monomeric form upon oxygenation, then it becomes necessary to show the change in (increase) in conductance upon oxygenation; conductivity apparatus (Radiometer) meter is being used.

Major Findings:

Influence of added urea in digioxin assay was studied because there are a number of reports on exceedingly high digioxin reported in the instances of patients on digioxin therapy and then had suffered a renal failure. In vitro test by radioimmune assay method (RIA) 5.0 mM urea would increase the assayable digioxin by a factor of two. On the other hand KCl was added up to 40 mEq/L with essentially no effect at all on assayable digioxin. However 10.0 mEq/L each of the following were investigated: NaCl, NaBr, NaI and NaSCN; NaCl had no effect at all but $Br^-$ $I^-$ and $SCN^-$ had essentially doubled the assayable digioxin, suggesting that hydrophobic interactions between serum albumin and digioxin could be weakened according to Hofmeister series.

Cell volume increase of red blood cells with HbS "sickling" was further investigated by a dipping refractometer and conductivity apparatus: index of refraction is higher when deoxygenated (sickled) and lower upon oxygenation (desickled). Upon oxygenation the process of depolymerization of Hb S molecules would expose electrically charged groups thus increasing the conductance in oxygenated red blood cell suspension -- the exposed electrically charged group gives rise to electrostriction; it is this that causes red cells to shrink upon oxygenation, and conversely conductance is indeed higher in oxygenated state.

Significance to NIAMDD Research:

Sickle cell anemia is a hereditary, hemolytic anemia -- an inborn error of metabolism, hence exceedingly significant to the NIAMDD mission. The root cause of human red blood cells (with HbS) "sickling" is due to hydrophobic interactions, which has been confirmed by over a dozen different laboratories. Murayama's theory has been tested at the bedside with success in Michigan as well as elsewhere (Santiago, Chile; Modevideo, Uraguay; Havana, Cuba; Mampong-Akwapim, Ghana; etc.).

Proposed Course of Project:

The main course of the project is focused on a "co-factor" of polymerization for Hb S. The usual course is to isolate and identify -- for which an adequate assay method is needed. We are in the process of working out a usable assay method.

Publications:

None.

| SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space) | U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT** | PROJECT NUMBER Z01 AM-27008-18 LPB |
|---|---|---|

PERIOD COVERED

July 1, 1976 through September 30, 1977

TITLE OF PROJECT (80 characters or less)

Studies of metabolic activity in microorganisms

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

| | | | |
|---|---|---|---|
| PI: | E. S. Kempner | Research Physicist | LPB NIAMDD |
| OTHER: | J. H. Miller | Chemist | LPB-NIAMDD |

COOPERATING UNITS (if any)

Dr. Vincent Bono, NCI
Dr. David Klein, NICHHD

LAB/BRANCH

Laboratory of Physical Biology

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Comparative Physiology Section

INSTITUTE AND LOCATION

NIAMDD, NIH, Bethesda, Maryland 20014

| TOTAL MANYEARS: | PROFESSIONAL: | OTHER: |
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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Studies of metabolic pathways in axenic cultures of microorganisms are continuing. The relationships between biochemically sequential reactions are being examined.



PHS-6040

Project Description:

Objectives:

An understanding of the control of metabolic activity in unicellular organisms.

Methods Employed:

Culture of microorganisms in the presence of radioactive or other compounds; chemical and physical fractionation of the cellular materials by ultracentrifugation, chromatography and chemical extraction. Microscopic examination of cells by time-lapse video micrography. Electronic measurement of cell volume distribution and analysis in terms of cell growth and age.

Major Findings:

Isolation of the shikimic acid enzyme complex from Euglena gracilis is being improved. The pathway catalyzes five sequential reactions leading to the final product, chorismic acid. Procedures have been established which achieve 100-fold purification in three steps with 50% yield.

Related studies of the metabolites involved in this pathway have also continued. Two-dimensional chromatographic procedures have been developed which allow the simultaneous separation of all six metabolites. Quantitative detection of all intermediates permits analysis of the facilitated and limiting reactions in a pathway.

Measurement of DNA in Euglena gracilis by flow microfluorometry was previously reported. This method is both rapid and sensitive, but unfortunately only gives relative values. In collaboration with Dr. Vincent Bono, NCI, measurements of cellular DNA were made in the protozoan, Euglena gracilis, and also in the murine leukemic strain L-1210. Both microfluorometric determinations of the DNA distribution in normal cell populations and also the average cellular DNA measured by classical chemical determinations (diphenylamine and indole reactions) were obtained. The computer-calculated distribution mean was set equal to the absolute value of average picograms DNA per cell.

Similar measurements made on cultures exposed to conditions that alter cellular DNA levels (colcemid and ethanol for mammalian cells, vitamin $B_{12}$ starvation for Euglena) showed that the quantitative change in DNA per cell measured by the two techniques was the same. Detailed studies of fluorescent stains (choice, technics of use, etc.), storage and treatment of stained cells, linearity of scale in flow microfluorometer, efficiency of staining in drug-inhibited cells, etc. were completed. Conditions were optimized for accurate, reproducible re-

sults. For the first time, the DNA distributions among entire populations of protozoa and mammalian cells have been rigorously established on an absolute scale.

Post-natal development of the rat pineal gland was studied in collaboration with Drs. D. Klein and M. Buda, NICHHD. At birth, isolated glands contain no HIOMT (Hydroxy indole-O-methyl transferase) activity, an enzyme in the serotonin pathway which ultimately leads to melatonin. Neither is innervation of the gland present at birth. Trypsinization of the tissue releases a homogeneous population of large cells (308 $\mu m^3$ volume) with large nuclei.

During the course of post-natal development, measurements of protein, DNA, HIOMT activity (and other pathway enzymes), leucine and thymidine incorporation, and cell volume were performed. The missing enzyme activity was measurable by age five days, and continued to increase linearly for 3 weeks. Cell volume increases continuously for three weeks, increasing about 50% on the average). Between 23 and 32 days there is an appearance of small cells (52.7 $\mu m^3$) which increase in number but not volume, while the proportion of large cells drops to zero. By age 35 days, only the small cells are seen and these persist throughout life.

Publications:

Kempner, E. S.: The changing nature of biophysical science. Biophys. J. 17: 203-204, 1977.

Shehata, T. E. and Kempner, E. S.: Growth and cell volume of Euglena gracilis in different media. Applied and Environ. Microbiol. In press.

REPORTED IN PRESS - 1976

Kempner, E. S.: Properties of organized metabolic pathways. Subcellular Biochem. 4: 213-221, 1976.

Miller, J. H. and Kempner, E. S.: The molecular biology of Euglena gracilis. X. Amino acid composition of protein. J. Protozool. 23: 444-446, 1976.

ANNUAL REPORT OF THE
LABORATORY OF BIOPHYSICAL CHEMISTRY, NIAMDD

## THE STRUCTURE AND ACTION OF PROTEINS

### TRANSGLUTAMINASE:

A. Fibroblasts: Tissue transglutaminase found in the supernate of sucrose extracted fibroblasts (after 100,000 X g centrifugation) of L929 mouse fibroblasts catalyzes the incorporation of $^{14}$C-methylamine into Z-gln-gly as well as casein. If labeled fibrinogen is added to this supernate, the fibrinogen is partially cross-linked. Certain proteins are present in the extract which seem to be specific substrates of the fibroblast transglutaminase. These proteins were partially purified by gel chromatography and the above observation was repeated. Labeled fibrinogen and actin could be coupled on to the surface of these fibroblasts by means of transglutaminase. First, the surface Iodination Pattern was determined, then an attempt was made to specify possible substrates of the transglutaminase catalyzed coupling of fibrinogen and actin. By the method we used, certain changes were observed: The quantity of some proteins decreased; the radioactivity on the top of the gels considerably increased. However, a definite answer was not found as far as the possible substrate or substrates are concerned. (Laki, Fésüs).

B. Antigenic sites: The immunization of rabbits with purified guinea pig liver transglutaminase resulted in the appearance of two antibody populations against the enzyme: one which reacted only with the $Ca^{2+}$-enzyme complex and another which reacted with the intact as well as the $Ca^{2+}$-enzyme. When the glutamine substrate was a dipeptide, the interaction of the $Ca^{2+}$-enzyme and its antibody was an apparent activation. However, when protein substrates were used, an inhibition was observed. Activation and inhibition led to the conclusion that the interaction of the $Ca^{2+}$-enzyme and its specific antibody is not only a limited steric hindrance of the active center but also a stabilization of the otherwise labile $Ca^{2+}$-enzyme. The antibody population reacting with both forms of the enzyme is inhibitory, probably due to a prevention of the $Ca^{2+}$-induced formation of the active enzyme. (Laki and Fésüs).

C. Actin: Actin was isolated from the acetone powder of L929 fibroblasts. The isolation was not possible unless washed; glycerinated cells were used. Otherwise, there are certain proteins which copolymerize or just simply coprecipitate with F-actin during the preparation procedures. Some of these proteins can be found in the 100,000 X g supernate and can cause gelation of cell extracts when the temperature, the divalent cation concentration is appropriate.

Fibroblast F-actin was totally cross-linked by guinea pig liver transglutaminase forming very high MW polymers. The G-actin, which was purified by gel chromatography from the cell extract, (almost 60-70% of the total actin content of the fibroblasts remains in monomer form under physiologic conditions), is not cross-linked by transglutaminase. On the other hand, $^{14}$C-methylamine could be incorporated into both G and F-actin by means of

transglutaminase. High MW polymer formation by F-actin was inhibited when the amine was present. (Laki, Fésüs).

FIBRINOGEN:

A. Primitive - (Salmon and Lamprey): Studies are in progress on the structure and reactions of the fibrinogens, of lamprey and salmon. Detailed sedimentation-equilibrium studies of lamprey fibrinogen in various buffers and 6M guanidine give a molecular weight of 350,000. Molecular weights of the isolated, reduced and carboxymethylated (A)$\alpha$, (B)$\beta$, and $\gamma$ chains via sedimentation-equilibrium in 6M guanidine yield 109,000, 73,000 and 48,500, respectively. Almost identical results are obtained by chromatography of these chains in 6M guanidine on sepharose. This fortifies our assumption that lamprey fibrinogen can be formulated as $[(A)\alpha, (B)_2, \gamma_2]$ with only a single (A)$\alpha$ entity. Our purest, plasmin-free lamprey thrombin rapidly clots and degrades lamprey fibrinogen. Various coagulating enzymes have been reacted with lamprey fibrinogen and the morphology of the resulting fibrins observed by electron microscopy. These fibrins show the characteristic cross-banding with an axial repeating distance of 230Å.

The presence of an exceedingly active plasmin in the plasma of migrating Columbia River salmon has been confirmed utilizing Trasylal, a protease inhibitor capable of inactivating plasmin. This plasmin is active against purified salmon fibrinogen at temperatures of -20°C. (Gladner, Murtaugh).

B. Modified by Methylation: The molecular aspects of the polymerization of fibrinogen was investigated by modifying the fibrinogen molecule. Methylation of the carboxyl groups of the aspartic and glutamic acid residues in fibrinogen was accomplished using dimethyl sulfate and the trimethyl oxonium ion. Upon methylation, the fibrinogen molecules aggregated resembling the normal fibrin clot, suggesting that the polymerization of fibrinogen requires the removal of the negative charges. (Osbahr).

C. Fibrinopeptides: The participation of the fibrinopeptides in the mechanism of vasoconstriction via stimulation of a smooth muscle receptor is under study. Alprenolol, known to affect the contractile process by competing with the binding sites of receptors, reversed the hemostatic effect of the peptides on the fibrinopeptide-sensitized arterioles of the bleeding rat tail. In addition, propranolol, a smooth muscle receptor blocking agent, further increased the elevated bleeding time. These findings indicate that the fibrinopeptides in hemostasis become attached to a smooth muscle receptor site initiating a chain of events that link the receptor with the contractile mechanism. (Osbahr).

HEMOGLOBIN:

A. Sickle-cell: Several new techniques for measuring the oxygen equilibrium of aggregating solutions of sickle hemoglobin have been explored. Two methods, one spectrophotometric and one gasometric, are presently under development. (Pumphrey, Minton).

A new method for measuring the minimum gelling concentration of solutions of sickle hemoglobin under quasi-equilibrium conditions has been developed and measurements of solubility and minimum gelling concentration under identical conditions are in progress. (Pumphrey).

B. Valence Hybrids: The ligand binding equilibria of synthetic and naturally occurring mutant valence hybrid hemoglobins have been analyzed in the context of model-independent thermodynamics and in the context of two conceptually distinct allosteric models. It has been found that a simple sequential model provides a unified interpretation of all of the data considered whereas a concerted two-state model of comparable sophistication cannot. (Minton).

C. Thermodynamic and Hydrodynamic Properties: A simple model has been developed which quantitatively accounts for the experimentally measured concentration dependence of the thermodynamic and hydrodynamic properties of hemoglobin in salt solution. This model treats the solution as a suspension of quasi-spherical hard particles with size, shape and mass close to the corresponding properties of the hemoglobin molecule. (Minton, Ross[LMB]).

D. Subunit Redistribution: The relationship between the redistribution of subunits in the tetrameric hemoglobin and binding of carbon monoxide is being studied. A theory has been proposed and the study of the binding of carbon monoxide and oxygen in mixtures of these gases is underway. (Saroff, Murphy [NBS]).

E. Oxidation: Studies on the oxidation of hemoglobin by ferricyanide and hydroxylamine at pH 7 were carried out by stopped flow as well as with the Cary spectrophotometer. Ferricyanide appears to oxidize only the deoxy form of hemoglobin while hydroxylamine oxidizes both forms. (Simpson).

CELL SURFACE RECEPTORS: Equilibrium and kinetic data on the binding of insulin to cell surface receptors have been analyzed. It has been found that a simple model based, on postulates rather different from those underlying previous interpretations, is capable of quantitatively accommodating all of the reported data. (Minton).

SLOW DEGRADATION OF HEMOGLOBIN AND THYROGLOBULIN: Purified hemoglobin A releases very small amounts of peptides. These peptides have been studied by first separation on an ion-exchange column and then by hydrolysis of material in the resolved peaks. Preliminary results indicate that if these peptides derive from hemoglobin (and not from some contaminating protein), then the peptides originate from the middle of the β chain. Thyroglobulin on standing releases iodine, most of which appears as iodide ion. A small percent of the released iodine occurs in material which emerges on an ion-exchange column prior to $T_4$ as three small unidentified peaks. (Saroff, Warner, Hartley [LNE]).

THYROID HORMONE BINDING: We have analyzed the data on the binding of thyroid hormone to human serum prealbumin and have proposed some reasonable models to rationalize these data. (Saroff, CE Branch).

| SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space) | U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT | PROJECT NUMBER Z01 AM 31000-17 LBC |
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PERIOD COVERED
July 1, 1976 through September 30, 1977

TITLE OF PROJECT (80 characters or less)

Proteins, enzymes and peptides involved in blood coagulation

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

| | | | |
|---|---|---|---|
| PI: | J. A. Gladner | Sr. Staff Investigator | LBC NIAMDD |
| OTHER: | P. A. Murtaugh | Res. Chemist | LBC NIAMDD |
| OTHER COLLABORATOR: | Howard Bladen | Microbiologist | LEP NIAMDD |

COOPERATING UNITS (if any)

| | | |
|---|---|---|
| S. I. Chung | Sr. Investigator | LB NIDR |
| M. S. Lewis | Investigator | LVR NEI |
| J. E. Halver | Sr. Scientist | Coll. of Fisheries, Univ. of Wa., Seattle, Wa. |

LAB/BRANCH
Laboratory of Biophysical Chemistry

SECTION
Macromolecules

INSTITUTE AND LOCATION
NIAMDD-LBC NIH Bethesda, Maryland 20014

| TOTAL MANYEARS: | PROFESSIONAL: | OTHER: |
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| 3 | 3 | 1 |

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Characterization of some major coagulation factors of lamprey blood, one of the most primitive vertebrates, was performed. This included elucidation of the molecular properties of lamprey fibrinogen, as well as its individual subunits, (chains), via sedimentation-equilibrium, column chromatography and gel electrophoresis. The kinetic-molecular properties give a molecular weight of 350,000 ± 12,000 and indicate an unusual structure. Thrombin-like enzymes were reacted with this protein and the resulting fibrins examined by electron microscopy.

Project Description:

Objectives: Major reactions leading to blood coagulation are centered on the protein-protein interactions of various blood and plasma factors. Complete understanding of these reactions must involve elucidation of the detailed structure of the proteins involved. The final phase of blood coagulation leads to formation of fibrin by the action of thrombin, followed by the cross-linking (ligation) by Factor XIII to form the actual physiological "blood clot". An understanding of these biomolecular mechanisms in lower vertebrates may uncover valuable "clues" which may be utilized to practical advantages in the case of clotting disorders in man.

Methods: Methodology stresses protein-enzyme fractionation, purification, and stringent characterization of all experimentally utilized materials. Enzyme mechanisms play a major role in these investigations; all aspects of such are employed. All methods of physical, analytical and enzyme biochemistry are utilized; for example, gel electrophoresis, enzyme kinetics, radioactive incorporation assays, ultracentrifugation, automatic amino acid analysis, etc. Where needed, new and novel methods are devised.

Major Findings: Further characterization of lamprey fibrinogen: As reported previously, in order to elucidate the cross-linking reactions of lamprey fibrins, it was mandatory to purify, stabilize and characterize the lamprey fibrinogen. The importance of lamprey fibrinogen is unique in that it is the most primitive of the available vertebrate species. We have succeeded in the isolation of a very stable, undergraded and storable preparation of this protein. (Gladner, Chung).

Detailed sedimentation-equilibrium studies were carried out on the native lamprey fibrinogen, as well as the reduced and alkylated chains in aqueous buffers and 6M guanidine. A mean molecular weight of 352,000 ± 12,000 was obtained in aqueous buffers using a $\bar{V}$ = 0.704 calculated from amino acid analysis. The reduced fibrinogen examined on SDS-polyacrylamide gels gave molecular weights of 110,000, 78,000, and 50,000 for the (A)$\alpha$, (B)$\beta$, and $\gamma$ chains, respectively. This would yield a molecular weight of 476,000 if the conventional $[(A)\alpha_2, (B)\beta_2, \gamma_2]$ structure were employed, a value too high to agree with the sedimentation data.

Lamprey fibrinogen in 6M guanidine gives a molecular weight of 281,000 via sedimentation-equilibrium; the reduced and carboxymethylated (A)$\alpha$, (B)$\beta$, and $\gamma$ chains shift to 87,000, 58,000, and 39,000, respectively, in this media. There is an anomalous increase in partial specific volume when both lamprey and bovine fibrinogen are placed in 6M guanidine. The calculated lamprey fibrinogen $\bar{V}$ shifts from 0.704 to 0.736 and to a similar, but lesser extent, for a bovine fibrinogen control. (The mechanism for this has not yet been elucidated). Utilizing these correction factors, the reduced and carboxymethylated (A)$\alpha$, (B)$\beta$, and $\gamma$-chains give molecular weights in 6M guanidine of 109,000, 73,000, and 49,000, respectively, in close agreement to the results obtained with SDS-polyacrylamide gels. Chromatography of these reduced and alkylated lamprey chains on columns of sepharose in 6M guanidine

gave values of 115,000, 76,000, and 50,000, respectively, for the (A)$\alpha$, (B)$\beta$, and $\gamma$-chains. Thus, three independent methods give identical molecular weights for the 3 species of chains. These results fortify our assumption that the lamprey (A)$\alpha$-chain exists as a single entity and that lamprey fibrinogen may be formulated as $[(A)\alpha, (B)\beta_2, \gamma_2]$. If 110,000 is subtracted from the calculated weight of 476,000, then the molecular weight of 365,000 is in close agreement with the results obtained by sedimentation-equilibrium of the native molecule.

Various molecular weights have been reported by another laboratory, especially for the $\alpha$-chain of lamprey fibrinogen. However, the fibrinogen was first converted to fibrin prior to isolation of the constituent chains. In our studies, we have found that plasmin-free human and lamprey thrombins clot lamprey fibrinogen; however, both thrombins degraded the $\alpha$-chain(s) of the resulting fibrin rapidly, even in the presence of a plasmin inhibitor. (Gladner, Lewis, Chung).

Lamprey fibrin fibers produced by the removal of fibrinopeptides A, B, or both, by the action of coagulating or thrombin-like enzymes, have been investigated by electron microscopy. The resulting fibrins appear to have similar properties to those of human, demonstrating the characteristic cross-banding with an axial repeating distance of 230Å. (Murtaugh, Bladen, Gladner).

Significance to biomedical research and the program of the Institute: Detailed investigations of the characterization of blood coagulating systems of lower species may yield critical reactions and anomalies which may be masked in humans. Malfunctions or genetic throwbacks in human blood coagulation mechanisms may be discernible on the basis of these studies. Any information concerning clot formation is of vital importance since "heart attacks" and "strokes" are responsible for the majority of fatalities in the United States. For instance, during the past year, factors of lower vertebrates have been utilized therapeutically to control coagulation anomalies in humans.

Proposed course of project: Efforts are in progress to further continue the projects described above. A major effort has been instituted to isolate and characterize lamprey Factor XIII as well as the previously reported highly active plasmin of salmon plasma.

Publications: None.

| SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space) | U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT | PROJECT NUMBER Z01 AM 31002-06 LBC |
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PERIOD COVERED

July 1, 1976 through September 30, 1977

TITLE OF PROJECT (80 characters or less)

Intermolecular Interactions in Physical Biochemistry

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

| | | |
|---|---|---|
| PI: A. P. Minton | Research Chemist | LBC NIAMDD |
| Coinvestigator: | | |
| J. G. Pumphrey | Staff Fellow | LBC NIAMDD |
| Cooperating units at NIH: | | |
| P. D. Ross | Research Chemist | LMB NIAMDD |

COOPERATING UNITS (if any)

NONE

LAB/BRANCH

Laboratory of Biophysical Chemistry

SECTION

Section on Macromolecules

INSTITUTE AND LOCATION

NIAMDD LBC NIH Bethesda, Maryland 20014

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

During the last year we developed a simple model which quantitatively accounts for the concentration dependence of the thermodynamic and hydrodynamic properties of salt solutions of hemoglobin. The ligand binding equilibria of valence hybrid hemoglobins have been analyzed and shown to be incompatible with a concerted two-state allosteric model.

Several new techniques for measuring the oxygen equilibrium of aggregating solutions of sickle hemoglobin have been explored. We have developed a new technique for measuring the equilibrium minimum gelling concentration of sickle hemoglobin solutions.

Equilibrium and kinetic data on the binding of insulin to cell surface receptors have been analyzed and reinterpreted.

Project Description:

Objectives:

1. To increase our knowledge about and understanding of the process by which sickle hemoglobin aggregates and disaggregates.

2. To explore the structural and energetic bases of cooperative ligand binding in hemoglobin and its modulation by effector substances.

3. To characterize the concentration-dependent properties of solutions of biological molecules in terms of specific (chemical) and non-specific (non-ideal) interactions between solute molecules.

4. To develop a comprehensive thermodynamic and kinetic description of the binding of hormones and other macromolecular ligands to cell surface receptors.

Methods:

Theoretical studies employ methods of classical and statistical thermodynamics together with computer techniques for fitting theoretical model functions to experimental data. Experimental studies during the past year have utilized techniques of visible spectroscopy and polarographic determination of oxygen concentration together with standard preparative and analytical techniques of protein chemistry.

Major Findings:

1. We have analyzed data on the concentration dependence of the osmotic pressure and apparent weight average molecular weight (as measured by sedimentation equilibrium) of hemoglobin solutions at concentration of up to 350 g/l. It was found that these data could be quantitatively described over the entire concentration range by a simple model which treats the solution as a suspension of hard quasispherical particles with no long-range interparticle interaction. The model particles which best account for the experimental data have approximately the same size, shape and mass as a hemoglobin molecule. The non-ideal contribution to the total thermodynamic activity of hemoglobin increases rapidly with increasing protein concentration. (Minton, Ross).

2. Data on the concentration dependence of the viscosity of hemoglobin solutions at concentrations of up to 350 g/l have been analyzed. It was found that these data may be quantitatively described by the generalized form of a relation commonly used to describe the concentration dependence of viscosity in suspensions of hard spheres. (Minton, Ross).

3. We have explored several methods for measuring the oxygen equilibrium of aggregating solutions of sickle hemoglobin. Methods for measuring oxygenation of normal hemoglobin are not applicable because high concentrations of hemoglobin are required and because gelation of hemoglobin in bulk solution

interferes with oxygen transport in the solution. Several methods of circumventing the problem were studied, including encapsulation of minute quantities of concentrated hemoglobin in artificial cells and liposomes, and dispersion of hemoglobin solution as microdroplets in paraffin oil emulsions. As the preparation of emulsions is simplest and most reproducible, we have developed two methods, one spectrophotometric and one gasometric, for measuring the amount of oxygen bound to hemoglobin as a function of the partial pressure of oxygen which is brought into equilibrium with the emulsion. These methods are currently in the process of being tested and standardized. (Pumphrey, Minton).

4. The interpretation of the minimum gelling concentration (MGC) of sickle hemoglobin solutions as conventionally measured is complicated by kinetic factors which tend to increase the measured value over the value expected at "equilibrium". A new method for measuring the MGC at equilibrium has been developed and is currently being used to collect data which may be compared to solubility measurements obtained under identical conditions. (Pumphrey).

5. The ligand binding equilibria of valence hybrid hemoglobins (synthetic or mutant species containing two ferric and two ferrous hemes) have been analyzed in a model-independent thermodynamic context and in the context of conceptually distinct sequential and concerted two-state allosteric models. The sequential model, but not the concerted model, appears to provide a unified interpretation of the effects of many types of heme alteration upon the ligand affinity of unaltered hemes. (Minton).

6. The kinetic assay of DeMeyts and coworkers has been analyzed. It is found that results which have been interpreted as evidence for negative cooperativity in binding of insulin and other macromolecular ligands to cell surface receptors may be quantitatively accounted for by simple models which do not invoke negative cooperativity. (Minton).

Proposed Course of Studies:

1. Continuation of the studies of oxygenation of aggregating solutions of sickle hemoglobin.

2. Extension of the hard particle model for nonideality to heterogeneous systems.

3. Continuation of the measurements of equilibrium MGC and solubility of sickle hemoglobin.

4. Measurements of the radio frequency dielectric dispersion of aggregating and non-aggregating solutions of sickle hemoglobin.

5. Analysis of the equilibrium and kinetics of thyrotropin and cholera toxin binding to cell surface receptors (in collaboration with L. Kohn and G. Lee, NIAMDD-LBP).

Significance to Biomedical Research and Programs of the Institute:

The analysis of nonideality in concentrated solutions of hemoglobin reported here constitutes a major step forward in our understanding of the energetics of the reactions of biomacromolecules under physiological conditions, i.e., in concentrated solution. Because of this work we know that nonideality must be taken into account if meaningful thermodynamic and kinetic interpretations of equilibria and reaction rates at high macromolecular concentrations are to be obtained. Moreover, the work provides the necessary methods for taking nonideality into account in simple systems containing one macromolecular species.

Heretofore, the mechanism of polymerization and depolymerization of sickle hemoglobin has been studied by means of an unphysiological cycle of heating and cooling fullydeoxygenated hemoglobin. The present study of oxygenation in aggregating systems represents the first attempt to explore the consequences of a more physiological cycle of oxygenation and deoxygenation at constant temperature upon the state of sickle hemoglobin in concentrated solution.

The study of ligand binding equilibria to valence hybrids has provided the most clear-cut evidence to date that the commonly accepted concerted two-state allosteric model cannot provide a unified explanation of cooperative ligand binding in hemoglobin.

Current studies of the binding of macromolecular ligands to cell surface receptors are not ordinarily analyzed with the attention to sources of error and uncertainty that characterize the best physical-chemical studies of binding equilibria and kinetics. It is important to apply a more rigorous analytical technique to these data so that one may ascertain whether the data support one particular interpretation or are consistent with a variety of interpretations.

Publications:

1. Minton, Allen P.: Quantitative relations between oxygen saturation and aggregation of sickle-cell hemoglobin: Analysis of oxygen binding data. In Hercules, J. I., Cottam, G. L., Waterman, M. R., Schechter, A. N. (Eds.), Molecular and Cellular Aspects of Sickle Cell Disease (DHEW-NIH Publication 76-1007, Bethesda, Md., 1976) pp. 257-273.

2. Minton, Allen P.: Non-ideality and the thermodynamics of sickle-cell hemoglobin gelation. J. Mol. Biol. 110: 89-103, 1977.

3. Fung, Leslie W.-M., Minton, Allen P., Lindstrom, T. R., Pisciotta, A.V. and Ho, C: Proton nuclear magnetic resonance studies of Hemoglobin M Milwaukee and their Implications concerning the mechanism of cooperative oxygenation of hemoglobin. Biochemistry 16: 1452-1462, 1977.

4. Ross, Philip D. and Minton, Allen P.: Analysis of Non-ideal Behavior in Concentrated Hemoglobin Solutions. J. Mol. Biol. 112: 437-452, 1977.

5. Ross, Philip D. and Minton, Allen P.: Hard Quasispherical Particle Model for Viscosity of Hemoglobin Solutions. Biochem. Biophys. Res. Commun. 76: 971-976, 1977.

| SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space) | U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT** | PROJECT NUMBER ZO1 AM 31003-03 LBC |
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PERIOD COVERED
July 1, 1976 through September 30, 1977

TITLE OF PROJECT (80 characters or less)
The action of thrombin on fibrinogen and a study of related blood clotting mechanisms.

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: A. J. Osbahr Res. Chemist LBC NIAMDD

COOPERATING UNITS (if any)
None

LAB/BRANCH
Laboratory of Biophysical Chemistry

SECTION
Physical Biochemistry

INSTITUTE AND LOCATION
NIAMDD NIH Bethesda, Maryland 20014

| TOTAL MANYEARS: | PROFESSIONAL: | OTHER: |
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☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The fibrinopeptides appear to be involved in hemostasis in the reaction that links the muscle receptor to the contractile mechanism. In the presence of alprenolol, which competes with the binding sites on muscle receptors, the hemostatic effect is reversed causing an increase in bleeding time of scarified rat tail arterioles. These results may be interpreted as receptor modification with a resultant decreased vasoconstriction, if indeed the fibrinopeptides act in the hemostatic mechanism by altering the muscle receptor site and thereby enhancing the biologic activity. The structural modification of fibrinogen by methylation of the carboxyl groups resulted in a polymerized network resembling the fibrin clot. This polymerized fibrinogen possesses similar properties as that of the fibrin polymer such as solubility in 6M urea and 2M acetic acid which is reversible through dialysis. These results indicate that the polymerization mechanism of fibrinogen involves the removal of negative repulsion, generated by carboxyl groups, enabling aggregation to occur. This system may serve as a model for the future characterization of the clinically undesirable physiological thrombus formation within blood vessels.



PHS-6040

Project Description:

Objectives:

Studies are being continued to increase our understanding of the structural basis for the physiological action of thrombin on fibrinogen and of the biological activity of the fibrinopeptides released from fibrinogen during blood coagulation. Further evaluation is in progress to gain insights into the possible role of these fibrinopeptides in hemostasis, vasoconstriction and pulmonary hypertension.

Methods:

Homogeneity and purity of the protein and peptide systems were evaluated with ultracentrifugation, regular and SDS gel electrophoresis, thin layer chromatography and paper chromatography. The peptide structure and composition were analyzed by high voltage electrophoresis, liquid column chromatography and spectrophotometric analysis.

Proteolytic enzymes were employed for "active site" determinations. Physiological monitoring was performed on rats by means of intracarotid cannulation. The hemostatic effect was followed by microscopic visualization of bleeding arterioles of scarified rat tail.

Major Findings and Proposed Course of Study:

The modification of fibrinogen was undertaken in the hope of gaining some insight into the mechanisms involved in the following reactions. 1. The polymerization of fibrin. 2. The enzymatic action of thrombin on fibrinogen. 3. The fibrinopeptide participation in hemostasis and vasoconstriction. The results of these findings may be presented as follows:

1) In an attempt to characterize the polymerization process, fibrinogen was modified by methylation with four distinct agents: diazo methane, thionyl chloride, dimethyl sulfate and the tri methyl oxonium ion. In the case of diazo methane and thionyl chloride, the reaction involved an SN2 substitution of a methyl group on the functional groups of the fibrinogen molecule. During this process, the modified fibrinogen polymerized into a fibrin-like clot in about two hours. Upon extensive proteolytic degradation of this modified fibrinogen polymer, it was observed via two dimensional thin layer chromatography that aspartic acid, glutamic acid, serine, histidine, tyrosine, lysine and arginine were methylated.

On the other hand, when dimethyl sulfate was employed for modification, polymerization occurred in fifty minutes and upon inspection of the product, most of the aspartic and glutamic acid residues were methylated along with a small amount of serine and histidine.

Modification via the tri methyl oxonium ion, which involved a nucleophilic attack by the SN2 mechanism of the carboxylate ion on the "oxonium

ion", resulted in polymerization within ten minutes. Examination of the modified product indicated the methylation of aspartic and glutamic acid residues and no detectable amounts of other modified amino acids.

In all cases the modified fibrinogen polymer resembled the fibrin clot. Its solubility in 6M urea and 2M acetic acid with reversibility through dialysis was identical to that of the fibrin clot.

Methoxyl determination indicated the modification of 118 aspartic and glutamic acid groups in the case of modification via the tri methyl oxonium ion. On the other hand, modification with diazo methane and thionyl chloride resulted in 350 to 400 groups being methylated.

It is indeed interesting that, if as we believe, the fibrinogen chains are kept apart by the high negative charge at the N-terminus of each chain, then upon neutralization of these charges, the resulting molecule would be expected to polymerize. The fibrinopeptides, which come from the ends of two of the three fibrinogen chains, possess high negative charge. It is only after the action of the clotting enzyme thrombin, which by limited proteolytic action hydrolyzes these negatively charged peptides from fibrinogen, that the resulting fibrin molecules polymerize. It is, therefore, reasonable to assume that before thrombic action, the fibrinogen chains possess mutual repulsion and are prevented from polymerizing by the high negative charge density in the vicinity of the attachment of the fibrinopeptides. The modification diminished this negative charge at the end of the fibrinogen chain by methylating the carboxyl groups of the aspartic and glutamic acid residues. This enabled the previously unpolymerized fibrinogen to aggregate without the intervention of the enzyme thrombin.

These current studies indicate by analogy that the mechanism of physiological clot formation involves, as a first step, the removal of negative repulsion at the ends of two of the fibrinogen chains via fibrinopeptide removal by thrombin, thus enabling the resulting fibrin to polymerize into the clot network.

2) The proteolytic action of thrombin on fibrinogen involves a limited proteolysis at an arginyl-glycyl bond in the fibrinogen molecule with the release of fibrinopeptides. Which factors influence this proteolytic action are of extreme interest in understanding the blood clotting mechanism. It is important to determine what influence the negative charges in the vicinity of the N-terminus of the fibrinopeptides has on directing the proteolytic action of thrombin. Methylation of fibrinogen was accomplished with the tri methyl oxonium ion and the resulting polymer was washed and purified. The modified fibrinogen was then incubated at 37°C with the enzyme thrombin and aliquots removed at various time intervals. Preliminary studies indicate that reduction of the negative charge by methylation decreased the enzymatic action of thrombin on fibrinogen. This strongly implies that the negative charge density in the N-terminus of the fibrinopeptides is a directing influence on the enzymatic action of thrombin on fibrinogen.

3) The methylation of the fibrinopeptides, in order to study their participation in vascular contraction and hemostasis, was accomplished by reaction of the peptides with dimethyl sulfate and the tri methyl oxonium ion. Separation of the peptides was achieved by chromatography on a Dowex 50 ion exchange resin. The extent of modification of each peptide was estimated by methyoxyl determination. Further studies on this project will be directed toward what effect this alteration in structure of the fibrinopeptides has produced on their vasoconstrictive properties.

Other significant findings are related to the participation of the fibrinopeptides in the mechanism of vasoconstriction by possibly linking the muscle receptor to the contractile mechanism. Alprenolol, which is known to participate in the contraction process by competing with the binding sites of receptors, reversed the hemostatic effect and increased the bleeding time of the peptide interaction with the bleeding arterioles. The bleeding time can be further increased by the addition of another component propranolol, a smooth muscle receptor blocking agent, which alters the structure of the "active site" of the receptor.

One may interpret these results as indicating that the injury of a vessel triggers a chain of reactions that terminate in a release of fibrinopeptide which is capable of acting on some smooth muscle receptor component of the vessel resulting in a stimulation or modification of the receptor site. This somehow alters its structure setting off a series of events, that link the receptor to the contractile mechanism, culminating in vasoconstriction. This process would decrease the flow of blood and effectively prevent the washing away of a partly formed clot until a more permanent protector of clot is generated.

Significance of Biomedical Research and the Program of the Institute:

Our studies indicate a relationship between the peptides from the blood clotting protein fibrinogen and the physiological processes in blood circulation. It is hoped that investigation of these physiologically active peptides may result in a deeper understanding of the mechanisms associated with hemostasis, vasoconstriction and blood coagulation; and would provide clues to the etiology of certain diseases associated with high blood pressure (hypertension), such as respiratory distress syndrome. The presence of fibrinopeptides may represent a possible agent for such vasoconstriction. The more we know about the physiological activities of these peptides the easier could be the task of sorting out some lesser known mechanisms involved in the related diseases.

The modification of fibrinogen by methylation producing a nonenzymatic polymerization may be used as a model to investigate the thrombin-induced polymerization of fibrinogen. This modification suggests a mechanism of clot formation by removal of the negative repulsion at the ends of two of the fibrinogen chains without peptide material being necessarily simultaneously released. This suggests a possible application in our future understanding

of clinically undesirable physiological thrombus formation within blood vessels. This is a vital step in solving the "problems" of hemostasis.

Proposed Course of Project:

Studies will be conducted to further investigate the modified fibrinogens to enable us to utilize them as models to increase our knowledge of the mechanisms involved in blood coagulation, hemostasis and vasoconstriction.

Further investigations will be made to characterize the similarity between the physiological clot and the modified fibrinogen polymer. This project will include an investigation of the ultrastructure of the polymers by subjecting them to electron microscopic examination.

Proteolytic enzymes will be utilized to investigate the structure of the modified fibrinogen. We will also employ SDS gel electrophoresis in an attempt to characterize the extent of modification of the chains of fibrinogen by methylation, as well as the participation of the modified chains on the various enzymatic cross-linking reactions. A separation of the modified chains of fibrinogen will be conducted to determine the extent of modification, as well as the enzymatic action of thrombin on the individual chains.

Additional studies will also be initiated on the action of thrombin on the modified fibrinogen to characterize the mode of action of the enzyme in its relationship to structure alterations.

The physiological role of native and modified peptides from fibrinogen will be further investigated to establish the role of muscle receptor sites in the mediation of the fibrinopeptide interaction with the contractile mechanism enabling vasoconstriction to occur.

In addition, continued chemical modification of specific functional groups in fibrinogen, as well as the fibrinopeptides, will be explored in order to gain greater insight into the relationship between structure, biological activity and enzymatic specificity.

Publications: None.

| SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space) | U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT | PROJECT NUMBER ZO1 AM 31004-06 LBC |
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PERIOD COVERED
July 1, 1976 through September 30, 1977

TITLE OF PROJECT (80 characters or less)
The Elucidation of the Structure and Interactions of Biologically Important Macromolecules.

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

| | | | |
|---|---|---|---|
| PI: | H. A. Saroff | Sci. Director | LBC NIAMDD |
| OTHER: | R. B. Simpson | Res. Chemist | LBC NIAMDD |
| | C. V. Warner | Staff Fellow | LBC NIAMDD |

COOPERATING UNITS (if any)
Clinical Endocrinology Branch, NIAMDD
Dr. Robert W. Hartley, Lab. of Nutrition and Endocrinology, NIAMDD
Dr. Randy Murphy, National Bureau of Standards

LAB/BRANCH
Laboratory of Biophysical Chemistry

SECTION
Macromolecules

INSTITUTE AND LOCATION
NIAMDD-LBC NIH Bethesda, Maryland 20014

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Oxidation of hemoglobin is being studied using ferricyanide and hydrolamine.

Cooperative binding systems involving aggregation, redistribution, subunit interactions and ligand interactions are being formulated and studied in hemoglobin and other proteins related to hormone action.

Slow degradation of hemoglobin and thyroglobulin is under investigation in order to define and explain the phenomenon.



PHS-6040
(Rev. 10-76)

Project Description:

Objectives:

The main objective of this laboratory is an elucidation of the structure of naturally occurring macromolecules based on the methods of physical chemistry. Macromolecules are studied primarily in solution and the emphasis is on the accumulation of data on the state of the molecule with respect to its environment. Cross features of structures as well as fine detail may be observed by studies.

Methods:

In our studies aimed at finer details of protein structure, the methods used involve the measurements of the binding of small molecules or ions to specific sites on the protein molecules, nuclear magnetic resonance (on model compounds, so far), optical rotatory dispersion measurements and other spectral techniques. For other properties of the protein molecules, we employ the ultracentrifugal, light scattering, electrophoretic and diffusion techniques.

Major Findings and Proposed Course of Studies:

A. Oxidation of hemoglobin: The oxidation of human and equine hemoglobins in 0.05M pH7 tris buffer has been studied by stopped flow as well as with the Cary spectrophotometer. With 10mM ferricyanide the half time of the absorbance change (first-order with a large excess of ferricyanide) is about 0.18 seconds. With 100 mM hydroxylamine, the absorbance change is also first order with a half time of about one second, so that for a given concentration, the ferricyanide oxidation is more than fifty times as fast as the hydroxylamine oxidation.

Antonini, Brunori, and Wyman: Biochemistry 4, 545 (1965) had found a half time of about 30 m sec. at pH 7 for the oxidation of deoxyhemoglobin by 1mM ferricyanide, and postulated that only the deoxy form of hemoglobin is oxidized by ferricyanide. Our half time for the oxidation by 1mM ferricyanide in air (1.8 seconds), i.e., about 1/60 the rate for deoxyhemoglobin, is in fair agreement with this postulate since the fraction of deoxyhemoglobin at atmospheric pressure of oxygen is in the neighborhood of 0.01.

With hydroxylamine the above postulate is evidently invalid, for with this oxidant, the rate in the absence of air is only about three times the rate at atmospheric pressure of oxygen.

At low concentrations of hydroxylamine (first noticeable below about 10mM) the course of the oxidation of oxyhemoglobin is not first order, slowing down as the reaction proceeds. That this is due to consumption of hydroxylamine by reaction at some part of the protein other than the iron, is shown by the resumption of the normal rate of absorbance change due to oxidation if a new addition of hydroxylamine is made to a reaction mixture which has

slowed down. By normal rate we mean the rate based on the rate at initial conditions and the concentration of unoxidized hemoglobin left when the new hydroxylamine is added. (Simpson).

B. Action of hemoglobin: The binding of oxygen to partially oxidized hemoglobin is similar to the binding of oxygen to hemoglobin which is partially saturated with carbon monoxide. The phenomenon observed with carbon monoxide is apparently due to the very slow dissociation of carbon monoxide liganded to hemoglobin. Equations for this kind of binding have been developed utilizing the idea of redistribution of subunits amongst tetrameric hemoglobin molecules. Experiments on the binding of oxygen in the presence of small amounts of carbon monoxide are underway with the intent of evaluating the parameters in our theoretical equations. (Saroff, Murphy/Nat. Bur. Standards).

C. Slow degradation of hemoglobin and thyroglobin: Sterile solutions of hemoglobin purified to remove the small amounts of peptidases present yielded some small peptides on standing. These peptides were separated by ion exchange chromatography and serial samples from the resolved peaks were hydrolyzed and analyzed quantitatively for amino acids. Preliminary results of the analyses done so far indicated that the peptides originated from the middle of the β-chain of hemoglobin. Since the amounts of material were quite small, additional studies are required to rule out contaminating material in the preparation. (Saroff, Warner, Hartley/LNE).

Sterile solutions of purified thyroglobulin generate, on standing, small amounts of dialyzable iodine and ninhydrin reacting material. The iodine was detected using the ceric sulfate-arsenous reaction. When analyzed by ion exchange chromatography, most of the dialyzable iodine emerged as inorganic iodide and a small amount emerged prior to the $T_4$ peak as three resolved and as yet unidentified components. (Saroff, Warner, Burkhardt/CE).

D. Hormone binding: Cooperative effects deriving from reasonable models involving site-site interactions and aggregation are being explored to rationalize data collected on hormone binding to prealbumin. (Saroff and CE Branch)

Significance to Biomedical Research and the Programs of the Institute:

Our emphasis is on the relationship between chemical properties and the structure of macromolecules. Once these properties are properly related, the attack on the relationship between structure and function can proceed on a solid basis. The proteins and macromolecules studied are either enzymes or those of interest in biological systems. Our systems are highly controlled with reference to the conditions of the protein environment and the biomedical significance of our findings derive from extrapolation to the biological system. Studies on the binding properties of protein systems related to certain hormones provide insight to possible modes of cooperative effects in hormone action.

Publications:

1. Saroff, H. A.: Spontaneous Degradation of Hemoglobin and Egg Albumin. Arch. Biochem. Biophys. In press 1977.

2. Cheng, S.-Y., Pages, R. A., Saroff, H. A., Edelhoch, H. and Robbins, J.: Analysis of Thyroid Hormone Binding to Human Serum Prealbumin by 8-Anilinonaphthalene-1-sulfonate Fluorescence. Biochemistry 16. In press 1977.

| SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space) | U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT** | PROJECT NUMBER ZO1 AM 31008-02 LBC |
|---|---|---|

PERIOD COVERED
July 1, 1976 through September 30, 1977

TITLE OF PROJECT (80 characters or less)
How proliferating tissues may avoid immune surveillance. The role of transglutaminase.

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

| | | | |
|---|---|---|---|
| PI: | K. Laki | Chief, Section on Physical Biochemistry | LBC NIAMDD |
| | L. Fesus | Visiting Fellow | |

COOPERATING UNITS (if any)

| | | |
|---|---|---|
| S. I. Chung | Res. Chemist, Section on Enzyme Chemistry | LB NIDR |

LAB/BRANCH
Laboratory of Biophysical Chemistry

SECTION
Physical Biochemistry

INSTITUTE AND LOCATION
NIAMDD LBC NIH Bethesda, Maryland 20014

| TOTAL MANYEARS: | PROFESSIONAL: | OTHER: |
|---|---|---|
| 1.0 | 1/3; 1/3 | 1/3 |

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

It was found that guinea pig liver transglutaminase produced two kinds of antibodies in rabbits. The action of these antibodies on the enzyme was studied.

Project Description:

Objectives:

We have reported earlier that antibody generated against transglutaminase retarded tumor (YPC-1) in mice. In order to understand this inhibition, it was important to study the properties of the antibody.

Major Findings:

The immunization of rabbits with purified guinea pig liver transglutaminase resulted in the appearance of two antibody populations against the enzyme: one which reacted with the $Ca^{2+}$-enzyme complex and another which reacted with the intact, as well as the $Ca^{2+}$-enzyme. The $Ca^{2+}$-induced conformational change of the enzyme molecule exposes a new antigenic determinant which initiates the production of a specific antibody population. When the glutamine substrate of the enzyme was a dipeptide, the result of the interaction of the $Ca^{2+}$-enzyme and its isolated specific antibody was an apparent activation of the catalytic activity. However, when protein substrates were used, an inhibition was observed. The characterization of the mechanism of the activation and the inhibition has led to the conclusion that the consequence of the interaction of the $Ca^{2+}$-enzyme and its specific antibody is not only a limited steric hindrance of the active center but, besides that, a stabilization of the otherwise labile $Ca^{2+}$-enzyme. The other antibody population reacts with both forms of the enzyme and its inhibitory effect, which has been observed in each assay, could be due to a prevention of the $Ca^{2+}$-induced formation of the active enzyme.

Significance to biomedical research and the program of the Institute:

Since transglutaminase seems to be involved in tumor proliferation, the inhibitors to this enzyme could shed light on tumor proliferation.

Proposed course of project:

We intend to further study the inhibiting action of the antibody and the effect of the antibody to the antibody.

Publications:

1. Fesus, L. and Laki, K.: The two antigenic sites of tissue transglutaminase. Biochemistry. In press.

| SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space) | U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT** | PROJECT NUMBER Z01 AM 31009-02 LBC |
|---|---|---|

PERIOD COVERED
July 1, 1976 through September 30, 1977

TITLE OF PROJECT (80 characters or less)
Transglutaminase in fibroblasts

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

| | | | |
|---|---|---|---|
| PI: | K. Laki | Chief, Section on Physical Biochemistry | LBC NIAMDD |
| | L. Fesus | Visiting Fellow | LBC NIAMDD |

COOPERATING UNITS (if any)
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NIAMDD LBC NIH Bethesda, Maryland 20014

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

It was found that fibroblasts (L-929, mouse) contain transglutaminase. The proteins that appear to be specific substrates in the cell have been partially purified. It was also demonstrated that fibrinogen could be coupled to the surface of fibroblasts by means of transglutaminase.

Project Description:

Objectives:

We found earlier that transglutaminase is involved in the proliferation of implanted malignant cells. The present experiments were aimed to see if normal cells contain transglutaminase and if fibrinogen and actin can be coupled to the surface of these fibroblasts.

Major Findings:

Tissue transglutaminase could be found in the supernate of the sucrose extracted fibroblasts (after 1,000,000 g ctf) of L-929 fibroblasts (mouse). It catalyzes the incorporation of $^{14}$C-methylamine into Z-gln-gly as well as casein. If labeled fibrinogen is added to this supernate, the fibrinogen is partially cross-linked following the characteristic pattern of the tissue transglutaminase cross-linking of fibrinogen. Incubating this extract alone in appropriate conditions, there are certain proteins which seem to be specific substrates of the fibroblast Tgase. These proteins were partially purified by gel chromatography and the above observation was repeatedly proven. Labeled fibrinogen and actin could be coupled on to the surface of these fibroblasts by means of Tgase. First, the surface iodination pattern was determined, then an attempt was made to specify possible substrates of the Tgase catalyzed coupling of fibrinogen and actin. By the method we used, certain changes were observed: The quantity of some proteins decreased; the radioactivity on the top of the gels considerably increased. However, a definite answer was not found as far as the possible substrate or substrates are concerned.

Significance to biomedical research and the program of the Institute:

It is increasingly apparent that coupling of fibrinogen to fibroblast surface by plasma transglutaminase, (Laki-Lorand Factor), is essential in wound healing. A similar role of tissue transglutaminase is indicated in tumor proliferation.

Proposed course of project:

Attempts will be made to isolate the proteins from fibroblasts that appear to be specific substrates for transglutaminase.

Publications:

1. Fesus, L. and Laki, K.: On coupling bovine fibrinogen to the surfa of malignant murine plasma cells by means of transglutaminase. Biochem. Biophys. Res. Commun. 72: 131-137, 1976.

| SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space) | U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT** | PROJECT NUMBER ZO1 AM 31010 01 LBC |
|---|---|---|

PERIOD COVERED
July 1, 1976 through September 30, 1977

TITLE OF PROJECT (80 characters or less)
Actin in fibroblasts

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

| | | | |
|---|---|---|---|
| PI: | K. Laki | Chief, Section on Physical Biochemistry | LBC NIAMDD |
| | L. Fesus | Visiting Fellow | LBC NIAMDD |

COOPERATING UNITS (if any)
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Physical Biochemistry

INSTITUTE AND LOCATION
NIAMDD LBC NIH Bethesda, Maryland 20014

| TOTAL MANYEARS: | PROFESSIONAL: | OTHER: |
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CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Actin was isolated from fibroblasts and some of its properties, especially its interaction with transglutaminase, studied.



PHS-6040
(Rev. 10-76)

Project Description:

Objectives:

It has been reported in the literature that the state of polymerization of cellular actin changes in malignant transformation. Our work aimed to study fibroblast actin in order to be able to follow the fate of actin after malignant transformation.

Major Findings:

Actin was isolated from the acetone powder of these fibroblasts. The isolation was not possible unless washed; glycerinated cells were used. Otherwise, there are certain proteins which copolymerize or just simply co-precipitate with F-actin during the preparation procedures. Some of these proteins can be found in the 1,000,000 g supernate and can cause gelation of cell extracts when the temperature, the divalent cation concentration is appropriate. (No further experiments were done to clarify the role of these proteins exactly, but should be done later).

Adding guinea pig liver transglutaminase to it, the fibroblast F-actin can be totally cross-linked: very high MW polymers are formed. The G-actin, which was purified by gel chromatography from the 1,000,000 g supernate of the cell extract, (almost 60-70% of the total actin content of the fibroblasts remains in monomer form under physiologic conditions), is not cross-linked by Tgase. On the other hand, $^{14}$C-methylamine could be incorporated into this actin by means of Tgase, like into F-actin. In the latter case, the high MW polymer formation was inhibited when the amine was present.

Significance to biomedical research and the program of the Institute:

Actin has been implicated as being involved in cell division; therefore, the study of cellular actin could lead to a better understanding of wound healing and tumor proliferation.

Proposed course of project:

We plan to study the proteins that coprecipitate with F-actin during the preparation procedure.

Publications:

1. Laki, K., Seel, M. and Ladik, J.: Conformation of triphosphate and lysine-triphosphate-arginine complex. J. Theor. Biol. In press.

2. Laki, K.: The long Hungarian tradition. Trends Biochem. Sci. 1: 242-244, 1976.

# ANNUAL REPORT OF THE LABORATORY OF MOLECULAR BIOLOGY

## NATIONAL INSTITUTE OF ARTHRITIS, METABOLISM AND DIGESTIVE DIESEASES

The work of the Laboratory of Molecular Biology is concerned with the understanding of biological processes in terms of structure and reactions at the molecular level. The areas of research include direct investigations of biological processes, such as genetic recombination DNA replication, and protein synthesis, studies of the mechanism of transformation by SV40 (a virus causing tumors in hamsters), and studies of the mechanism of hemoglobin S gelation, the phenomenon which causes sickling of red blood cells in sickle cell anemia. Other investigations are concerned with the structure and chemical properties of biologically important materials. These include studies of the organization of DNA and proteins in chromatin, investigations of protein structure by x-ray diffraction, and of nucleic acid and polynucleotide structure both by diffraction and spectroscopic methods, and theoretical studies of the mechanisms of free energy transduction, muscle contraction, membrane properties, and biochemical kinetics. Important results have been obtained in the laboratory in a number of these areas during the past year.

### Study of Functions involved in Genetic Recombination

We have identified an enzyme activity that introduces superhelical turns into covalently closed circular DNA. This enzyme, DNA gyrase, has been extensively purified from E. coli cells. The reaction requires ATP as cofactor. The DNA product is negatively supercoiled; this is the same direction of supercoiling found in all covalently circular DNA isolated from cells.

DNA gyrase is specifically inhibited by coumermycin and oxolinic acid. In both cases, enzyme from a drug-resistant mutant strain is insensitive to the drug. The two drug-resistance loci are genetically distinct, which leads us to conclude that each locus is responsible for one subunit of the enzyme. As both drugs are known to function as inhibitors of DNA replication, DNA gyrase must play an essential role in this process.

### Replication of Colicin E1 Plasmid DNA

Replication of closed-circular colicin E1 plasmid (Col E1) DNA can be initiated and completed in extracts of Escherichia coli. The major products of in vitro replication are completely replicated molecules and a unique type of early replicative intermediate containing a newly synthesized DNA fragment(s) in a small replication loop. The fragment has an average length of approximately one fifteenth of the unit length of the plasmid molecule and has a sedimentation constant of approximately 6 S. Synthesis of the intermediates is inhibited by rifampicin but most of the intermediates can complete replication in the presence of rifampicin. The first synthesis of a DNA fragment is initiated at a specific site on the H strand and depends on the function of E. coli DNA-dependent RNA polymerase. Subsequent synthesis of the DNA fragment on the L strand does not involve the RNA polymerase.

Cleavage maps of colicin E1 plasmid DNA and its smaller derivative, pNT1 DNA, were constructed using restriction endonucleases. The nucleotide sequence

of a region which contains the origin of replication was determined. The site of the nucleotide from which DNA replication is initiated was determined with 6S L-fragments, which is the DNA fragment first made on Col E1 DNA. The fragments were labeled with [$\gamma$-$^{32}$P]ATP and polynucleotide kinase at the 5'-OH groups which were uncovered by alkali treatment. The site is one of three consecutive nucleotides, dA, dA, and dC located at a unique position. One or a few rA residues were found to be attached to some of the DNA molecules. The transition from the primer RNA to DNA occurs in a region consisting of a segment of five A residues. Both sides of this segment are very rich in G and C. This is the first case where the origin of DNA replication is determined at the precise location in the nucleotide sequence.

Attachment of RNA to 6S L-fragments was shown by the transfer of $^{32}$P-label in the DNA derived from one of the [$\alpha$-$^{32}$P]dNTPs to one of 2'(3')rNMP after alkali hydrolysis of 6L-fragments. It was found the rA-dA and rA-dC linkages predominate ribonucleotide-deoxyribonucleotide linkages in the 6S L-fragments. This result is in complete agreement with the expectation based on the analysis of the nucleotide sequence of the region that contains the origin of replication.

In vitro Col E1 DNA replication is carried out by bacterial functions. Purification of enzymes that participate in replication and reconstruction of a replication system by combination of purified enzymes are studied. DNA gyrase was found to be one of the essential enzymes and the gene responsible for resistance to coumermycin was identified as a structural gene for the enzyme.

## Mammalian Enzymes Involved in Repair or Replication of DNA

The aim of this project is to identify and characterize enzymes involved in the repair and replication of DNA in higher organisms. Currently we are 1) Surveying eukaryotic souces for a DNA gyrase-like activity, 2)Characterizing a DNA kinase or unusual specificity also from eukaryotic sources, 3) Examining the number and characteristics of DNA ligases in rat liver.

## Novel Recombination Systems

The Tn9 transposon - a genetic element which carries a gene for chloramphenicol-resistance flanked on both sides by IS1 (an insertion sequence) - possesses two remarkable features. 1) It is able to insert itself as a unit into a variety of unrelated chromosomes and at many different sites. 2) It can generate deletions of portions of itself and/or of adjacent chromosomal DNA. Satisfactory understanding of the genetic parameters of the latter activity has yet to be achieved. Part of the problemzis that Tn9 can be so "plastic" that standard genetic crosses frequently do not yield expected progeny.

## Genetics and Structure of the Oncogenic Virus SV40

We have demonstrated that the tumor specific transplantation antigen (TSTA) associated with SV40 transformed and lytically infected cells, copurified with the tumor antigen (TA). In addition the time course of appearance of TSTA parallels that of TA in lytic infection; and TSTA and TA are present in most tsA mutant-transformed cells, although the antigens disappear coordinately in one rat line. We have postulated that the two antigenic activities represent different antigenic sites of the same polypeptide.

The distance between origins of DNA replication in normal and SV40 transformed cells have been analyzed. In depleted medium, nontransformed cells enter a resting state and stop DNA replication. Those few cells which continue DNA replication after 24 hrs in depleted medium (1-3% of the population) had an interorigin "average" distance of 40 μ. In complete medium, where 50% of the nontransformed cells replicate their DNA, the distance was also 40 μ. In depleted medium, transformed cells do not enter a resting state and at 24 hr of depletion, still ∿ 50% of the cells continued DNA replication. Under these conditions, the "average" distance between origins of DNA replication increased to about 60 μ. In complete medium, the distance between origins of DNA replication in transformed cells dropped to about 30 μ. We have interpreted these results as indicating that in normal cells, initiation is the result of a normal initiation protein which recognizes sites on the DNA, separated by ∿ 40 μ. In transformed cells in depleted medium, no normal initiation protein is synthesized, but TA acts as an initiator by recognizing new DNA sites separated from each other by ∿ 60 μ. In complete medium, DNA synthesis is initiated in transformed cells by both the normal initiator and by the TA, hence the decrease in interorigin distance to 30 μ.

A model for the nucleoprotein core of SV40 virions has been constructed based upon the proposed structure of nuclesomes.

Protein Synthesis in Mammals: Mechanisms and Metabolic Controls

The long range objective of this project is to understand at the molecular level how proteins are synthesized in mammals and how the process of protein synthesis is controlled at the levels of translation and transcription. The biosynthesis of the human placental polypeptide hormones, human placental lactogen (hPL) and human chorionic gonadotropin (hCG), in cell-free systems is being studied as one approach toward this goal. We have isolated biologically active messenger RNA from both early and term human placentas and from cells derived from human cancers known to excrete hCG or its subunits. These mRNAs have been translated in the cell free system from wheat germ and some of the proteins synthesized which have antigenic determinants in common with hPL or hCG have been characterized. Poly(A) mRNA from term placenta directs the synthesis of two immunoprecipitable forms of hPL distinguishable on SDS polyacrylamide gels. The minor species co-electrophoreses with authentic hPL while the major precursor-like species has an apparent molecular weight of about 3000 greater. In addition to these two species, very high molecular weight forms of hPL ($\bar{M}$ > 150,000) are present in untreated cell-free reaction mixtures. Both the precursor-like material and the very high molecular weight aggregates synthesized *in vitro* bind to specific lactogen receptors on liver membranes, thus fulfulling the first requirement for biological activity. Early placental mRNA appears to direct the synthesis of material with antigenic determinants in common with hCG, but with a molecular weight considerably greater than either subunit of the hormone.

Structural Features of mRNA and their Influence on Translation

The aim of this project is to determine the influence of specific structural features of the 5'-terminal portion of eukaryotic mRNA on initiation of protein synthesis *in vitro*. By comparing the ribosome-binding efficiencies of mRNA's

containing different 5' ends the following conclusions have been reached: 5'-terminal $m^7G$ has a strong promoting influence on mRNA selection by eukaryotic ribosomes. The presence of the 2'-O methyl group as well as the nature of the penultimate residue also have a positive influence at this step, under mRNA excess conditions.

Thermodynamics of Hemoglobin S Gelation

The polymerization of hemoglobin S molecules into fibers and alignment of these fibers to form a crystalline phase (gelation) is responsible for the deformation (sickling) of the red blood cell in sickle cell anemia. The interpretation of both kinetic and equilibrium experiments on the gelation of hemoglobin S requires thermodynamic information.

Measurements of the solubilities and heats of gelation (8-42°C) have been interpreted in terms of a two-component, two-phase model corrected for the effects of protein non-ideality. At 37°C, gelation is a wholly entropically driven process and values of the standard state thermodynamic parameters have been estimated to be: $\Delta G^\circ = -3$ kcal $mol^{-1}$, $\Delta H^\circ \cong 0$ kcal $mol^{-1}$, $\Delta S^\circ \cong 10$ e.u.

The osmotic pressure and sedimentation equilibrium of hemoglobin solutions are accounted for by a model which treats the solution as a suspension of hard quasispherical particles. Non-ideality, due mainly to the protein excluded volume, makes a large contribution to the thermodynamic activity at the hemoglobin S concentrations at which gelation takes place. The tabulated activity coefficients resulting from this analysis will be needed for the proper interpretation of the kinetics of gelation and of the solubility behavior of hemoglobin mixtures.

The concentration dependence of the viscosity of hemoglobin molecules in neutral salt solution has been accounted for by relations describing the viscosity behavior of suspensions of uncharged macroscopic spheres. This result is consistent with the above view of the hemoglobin solution as consisting of hard quasispherical particles.

A systematic study of the effects of ions and organic molecules upon the solubility of deoxyhemoglobin S has been carried out. From these results and the current understanding of the structure of the HbS polymer we have proposed a stereochemical mechanism of the noncovalent inhibition of gelation of deoxyhemoglobin S. This model accounts for the effects of all inhibitors reported to date and suggests chemical modifications that will be required to achieve more effective inhibition.

Thermodynamics of Ligand Binding to Dehydrogenases

The similarity of the three-dimensional structure in the NAD-binding domain within the family of dehydrogenases is a familiar concept. An attempt has been made to correlate the structure-function relationship to the thermodynamics of coenzyme binding to several dehydrogenases. The $\Delta G^\circ$, $\Delta H^\circ$ and $\Delta S^\circ$ parameters are broadly similar for the binding of NAD(H) to yeast alcohol dehydrogenase, beef heart and rabbit muscle lactic dehydrogenases and pig heart

malate dehydrogenase differing only within a range of 2 Kcal/mol in $\Delta H^\circ$ and compensated by corresponding entropy changes resulting in minimal differences in $\Delta G^\circ$. However, in the case of horse liver alcohol dehydrogenase the $\Delta H^\circ$ values of binding of NAD and NADH are close to zero, the contribution to the binding coming solely from entropic changes. This is suggestive of conformational changes in the enzyme upon binding NAD(H). This view is further confirmed by the thermodynamic parameters of ADP-Ribose binding to the enzymes wherein the abnormality with respect to liver alcohol dehydrogenase vanishes. This study confirms the conclusion about the commonality of the topography of the NAD-binding domain in several dehydrogenases, and establishes that thermodynamics can reflect structure-function correlation with respect to ligand binding.

Chromatin Structure and Function

Chromatin is the DNA-protein complex present in the nucleus of higher organisms. During the past year, studies of chromatin structure have continued. Further evidence was obtained that the arginine-rich histones, H3 and H4, play a central role in the organization of chromatin subunit (nucleosome) structure. The organization of the DNA within the nucleosome has been studied; the pattern of discrete DNA fragments generated by nuclease attack has been shown to reflect a common set of recognition sites for all nucleases so far studied, spaced at 10 nucleotide intervals along the nucleosomal DNA.

The structure of transcriptionally active regions of chromatin is also under investigation. Nuclease and protease probes have been used to study chromatin structure in globin genes of avian reticulocytes. The organization of the DNA in these genes appears to involve nucleosomes with a structure similar to that present in inactive chromatin. Studies of transcription in model systems suggest that nucleosomes do not necessarily impede the progress of RNA polymerase. It is likely that the transcription mechanism in eukaryotes involves sliding, exchange or unfolding of nucleosomes.

Three Dimensional Structures of Proteolytic Enzymes

1) The structure has been determined of the acid protease from Rhizopus chinensis, an enzyme that is closely related to the mammalian enzymes pepsin, renin, and cathepsin D. A 2.5Å map was calculated and a model of the enzyme was traced, using the computer graphics system at the University of North Carolina. The model is now in a suitable state for refinement to 2Å resolution.

The molecule is bilobal with a pronounced cleft in which are found the two aspartic acid residues known to be involved in the enzymic activity. Inhibitors such as pepstatin have been shown to bind in the cleft and support the concept of extended subsite binding in the acid proteases.

The structure has been shown to be very similar to those of two other mold proteases, from J. penicillium and E. parasitica. Examination of the structure and comparison with the pepsin sequence indicates that it probably closely resembles pepsin.

2) The structure of γ-chymotrypsin has been continued.

Immunoglobulin Structure and Antibody Antigen Interaction

1) The 6Å electron density map of the human IgG (κ) immunoglobulin has been examined in the light of our present knowledge of the structures of antibody domains. The evidence for domain structure invariance is now quite strong and has led to the development here of a search technique for immunoglobulin electron density maps in which the location and orientation of particular domains can be determined with precision. In this way, the quaternary structure of the Dob molecule has been determined, representing the first intact immunoglobulin structure to be worked out.

The molecule is T-shaped with the 2 Fab arms extending roughly at right angles to the Fc, the latter having a very similar structure to that of isolated Fc. The angle between the constant and variable regions in the Fab is intermediate between that observed previously for other Fabs, and emphasizes the flexibility in the switch region of the molecule, in addition to the generally accepted hinge flexibility. The carbohydrate moiety plays an important role in the interactions between the Fab and the Fc.

2) A model has been proposed for the binding site of MOPC 315, a DNP binding mouse myeloma protein, which can account for the results of published ligand binding studies in solution. This model has since served as the starting point for further ligand binding studies by esr and nmr techniques.

Structural Studies on Polynucleotides

The aim of this project is to obtain structural information on polynucleotides and to provide insights into the factors which control structure, principally through X-ray fiber diffraction and model building studies. Current areas include 1) the structure of the poly U-spermine complex, 2) the structure of a "denatured" polynucleotide, poly A in denaturing solvents, 3) Prediction of structural parameters from observed helical parameters.

A·I and A·G Polynucleotide Pairing: Controlling Effect of NH Hydrogen Bonds to Solvent Water

A major objective of this project has been determination of the ways in which hydrogen bonding of nucleic acid bases affects the specificity and stability of polynucleotide interaction. In the past, we have examined direct effects in which hydrogen bonding is enhanced by addition of amino groups in appropriate positions or by direct blocking with bulky alkyl residues.

In the present study we have investigated an indirect effect of hydrogen bonding, which is mediated by the energetics of the helix-coil equilibrium. A·I pairs are known to form, but A·G pairs have not been observed. There have been contradictory predictions that such pairs will form (Rich) and that they will not (Crick). An acceptable pair of hydrogen bonds can be formed between A and G, but one proton of the G amino group would then not be able to make a hydrogen bond to solvent water because of steric exclusion of water

by proximity of 2-CH of A. While lack of this hydrogen bond would clearly be destabilizing to the helix, less complete stacking of the coil would presumably favor the helix. Since present knowledge of the energetics of the equilibrium does not permit a reliable prediction, an experimental approach to the problem was necessary.

The problem of A, G interaction cannot be usefully examined by mixing poly(A) with poly(G) because of the highly stable self-structure of G. We have investigated the same general principle, however, by observing the interaction of poly(I) with poly 2-aminoadenylic acid (poly($2NH_2A$)), in which the 2-substituents of A and G are transposed. Neither homopolymer in this case has a self-structure sufficiently stable to block hetero interaction. The prediction is that if inability of an NH proton to hydrogen bond to water in the helix can prevent helix formation, poly(I) and poly($2NH_2A$) will form a 1:1 complex (or possibly not interact at all). If the H-bond to water cannot play such a role, however, the two polymers should form an $AI_2$ triple helix.

Experiments showed that a stable helix is formed, and detailed examination of the stoichiometry demonstrated that a 1:1 complex was formed and that a 1:2 complex was not. Assurance that the observed blocking of A·I interaction at the pyrimidine (N1) bonding site of A did not result from direct steric blocking of I by the 2-amino group was provided by studies with poly 2-methyladenylic acid (poly(2MeA)). The $CH_3$ group is slightly larger than $NH_2$ but is not a hydrogen bond donor. If the $CH_3$ (or the $NH_2$) group is large enough to block pairing, then poly(I) would form only a 1:1 complex and not a triple helix. In this case, experiments demonstrated that only a 1:2 $AI_2$ complex is formed. We conclude that inability to form a hydrogen bond to water can control the direction of the equilibrium and prevent helix formation. An A·G pair is therefore not expected to form.

In the course of this work, it was also necessary to reinvestigate interaction of poly(A) with poly(I) and show that a prior report of a 1:1 complex of these polymers was erroneous. Only a triple helix is formed.

These experiments also led to a surprising conclusion about relative stabilities of N1 and N7 bonding sites in the $AI_2$ helix. Bonding at N7 is markedly more stable than at N1 in contrast to the A, U system where the opposite is true. These observations are of interest in connection with the wobble A·I pairing of mRNA to the anticodon.

## Structure of Poly(8BrA), an All-Syn Polymer

In order to study the effects of the unusual *syn* conformation on polynucleotide structure, we had previously synthesized the first polynucleotide in which all of the base residues were constrained to be *syn* (glycosidic angle $\chi \cong 240°$) by bulky Br residues. The polymer was shown to exist as a highly stable double helix with extensive base stacking. In order to obtain more detailed information of the structure, the study was continued with a combination of theoretical and experimental methods in collaboration with Dr. Girjesh Govil (LCP; permanent address: Tata Institute for Fundamental Research). Potential energy calculations (CPF) by Dr. Govil produced a set of

possible structures having relatively low non-bonded interaction energy. These candidate structures were then examined for consistency with experimental evidence (NMR, IR, UV) and for suitability as components of a hydrogen-bonded double helix. Many of the low energy structures would have placed the phosphates inside the helix and bases outside and were thus unsuitable for electrostatic and hydrogen bonding reasons. Others had C2 endo sugar conformations, excluded by NMR evidence. A single structure survived the exclusion process. Further refinement of this structure showed that only slight changes were required to obtain a very satisfactory double helix having parallel strands, stacked bases and a 2-fold rotation axis parallel to the helix axis. Of three possible hydrogen bonding schemes only a symmetrical one bonded at N1 and an amino proton is capable of satisfying the geometrical requirements. Each strand has C3 endo geometry and $\phi' = 216°$, $\omega' = 280°$, $\omega = 294°$, $\phi = 179°$, $\chi = 243°$, and $\psi = 57°$. Such a conformation leads to approximately 8 nucleotide units per turn of the helix and an axial rise of 3.9 Å.

One of the most interesting aspects of the results obtained in this study is that although the orientation of the base and the hydrogen-bonding scheme are different in the cases of poly(A) and poly(8BrA), their backbone conformations are very similar, both in the ordered and random-coil forms. These results provide further evidence that (a) the basic nucleotide structure is stabilised by short-range interactions within the backbone itself, (b) intra- and inter-strand base-base interactions (stacking and hydrogen bonding) provide additional stabilization to the structure corresponding to the conformational angles found in ordered RNA, and (c) a major fraction of a randomly coiled single-strand has a conformation similar to that of each strand in a double helix.

Ion Transport Across the Nerve Axon Membrane

In our previous work, we were concerned with the question of how to calculate the concentration noise power spectrum of linear kinetic systems when the rate constants of the systems are assumed to be known and the question of how to evaluate the rate constants of the reactions when adequate noise data are available. In our current work, we have extended our analysis to systems with non-linear kinetic reactions. Non-linear chemical reactions are very common in biochemistry. For example, the association-dissociation reactions of multisubunit systems such as hemoglobins and oligomeric enzymes, or most enzymatic reactions are non-first order reactions. In general, with the use of a "quasi-linearization" approximation, the phenomenological relaxation matrix, the variances, and the resulting noise power spectrum are all expressed in terms of the usual kinetic rate terms of the chemical mass action law. Thus, the calculation of the noise power spectrum becomes very simple as soon as the rate constants of all elementary reactions of the system are known. The formalism can be used as well to evaluate the rate constants of the elementary reactions of an arbitrary system if adequate noise data are available. Finally, the formalism is very useful in differentiating between an equilibrium and a non-equilibrium steady-state system. Applications to membrane channels such as potassium (or sodium) channels in nerve membranes and ionic channels in lipid bilayers have been discussed.

Energy Conversion in Biology, especially Muscle Contraction

The main accomplishment during the past year has been the completion and publication of a research monograph on the theory of free energy transduction in biology. This gives a unified account of the various well-known examples of free energy transduction such as active transport, muscle contraction, oxidative phosphorylation, etc. The relations between steady-state kinetics and thermodynamics are emphasized.

Work on explicit models of muscle contraction continues. The general approach we are using is set out in a paper with Evan Eisenberg. We hope to conclude the modelling work during the next year.

Extensive mathematical analysis has been carried out on the steady-state kinetics of the respiratory chain enzymes. The latter part of this work, to be published in the near future, was in collaboration with Britton Chance. The same analysis is also applicable to single-file diffusion through membrane channels.

A new project is a study of the effect of enzyme-enzyme interactions on steady-state enzyme kinetics. This generalizes the classical solution and lattice interaction problems of statistical mechanics from equilibrium to steady state. This could be a very extensive area to investigate.

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PERIOD COVERED
July 1, 1976 through September 30, 1977

TITLE OF PROJECT (80 characters or less)

Study of Functions involved in Genetic Recombination

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

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| | Mark Guyer | Staff Fellow | LMB NIAMDD |
| | Mary O'Dea | Chemist | LMB NIAMDD |
| | Michiyo Mizuuchi | Guest Worker | LMB NIAMDD |

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

We have identified an enzyme activity that introduces superhelical turns into covalently closed circular DNA. This enzyme, DNA gyrase, has been extensively purified from E. coli cells. The reaction requires ATP as cofactor. The DNA product is negatively supercoiled; this is the same direction of supercoiling found in all covalently circular DNA isolated from cells.

DNA gyrase is specifically inhibited by coumermycin and oxolinic acid. In both cases, enzyme from a drug-resistant mutant strain is insensitive to the drug. The two drug-resistance loci are genetically distinct, which leads us to conclude that each locus is responsible for one subunit of the enzyme. As both drugs are known to function as inhibitors of DNA replication, DNA gyrase must play an essential role in this process.



PHS-6040
(Rev. 10-76)

Project Description:

DNA Supercoiling - (Gellert, Mizuuchi, O'Dea)

Over the last two years we have made some progress in understanding the biological origin, and biological function, of DNA supercoiling. Supercoiling is a property of DNA molecules whose strands are joined, head to tail, to form a covalently joined structure (covalently closed circular DNA, or ccc-DNA for short). Such molecules are easily made by enzymatic sealing in vitro. One way of making supercoiled molecules is to change the average pitch of the DNA double helix from what it was when the molecule was sealed to form the ccc-DNA; small changes can be produced by varying the salt concentration or temperature, larger changes by the binding of certain proteins such as histones, or of dyes such as ethidium bromide which insert themselves between the DNA bases. Because the number of times that the two strands wrap around each other is locked in at the time of sealing and cannot change, the double helix must then coil around itself (supercoil) to compensate.

Supercoiled DNA is widely distributed in nature. It is found in certain viruses (e.g. polyoma, SV40, phage PM-2); most plasmids are supercoiled, and even entire chromosomes (the genome of E. coli is a giant supercoiled molecule). It turns out, in fact, that ccc-DNA isolated from any type of cell is always supercoiled, and always in the same direction. The DNA acts as if it had too few Watson-Crick turns for its total length; it is termed underwound, or negatively supercoiled.

Because a DNA molecule resists being twisted around its axis, in much the same way as a rubber hose does, considerable amounts of energy can be stored in the supercoiled conformation. Take as an example the covalently circular form of phage $\lambda$ DNA (MW $\simeq 3 \times 10^7$): as extracted from E. coli, it has about 1000 kcal/ mol of supercoiling free energy.

Until recently, it was thought that ccc-DNA was nevertheless in a relaxed conformation inside cells, and that the supercoiling associated with purified ccc-DNA reflected the difference between the surroundings of the DNA inside and outside the cell. In this model, supercoiling would be caused by removal of proteins and other substances which had perturbed the structure of DNA inside the cell; in a sense, supercoiling would be an artifact of extraction of DNA.

It now appears that the origins of supercoiling are more specific and interesting. At least in bacteria, DNA is actively supercoiled in the cell, and supercoiling turns out to be an essential step in DNA replication as well as in other functions of DNA.

Several lines of evidence have led us to this view. First, we have isolated from E. coli an enzyme (DNA gyrase) that introduces superhelical turns into DNA in an ATP-driven reaction. The turns are in the same direction as in DNA extracted from cells, and the final number of turns is comparable

or slightly greater. The enzyme has a molecular weight of 140,000 and is made up of two subunits whose molecular weights are roughly 45,000 and 95,000. It is thus the simplest biological system so far found to be capable of mechanochemical coupling.

Evidence about the biological role of DNA gyrase comes from studies on antibiotic sensitivity. The enzyme is inhibited by low concentrations of novobiocin and the related drug coumermycin, but gyrase from a coumermycin-resistant mutant of E. coli is resistant to both drugs. Thus DNA gyrase seems to be the biologically significant target of these antibiotics. Novobiocin and coumermycin are known to inhibit the replication of the E. coli chromosome, as well as the replication of several types of double-stranded ccc-DNA (colicin E1 plasmid, $\phi$X-174-RFI) in cell-free systems derived from E. coli. We thus conclude that DNA gyrase supplies an essential function for the replication of these molecules. In agreement with this interpretation, cell-free replication of colicin E1 DNA can be partly rescued from novobiocin inhibition by the addition of purified resistant DNA gyrase.

Why should DNA replication show a requirement for a supercoiling activity? As a ccc-DNA molecule replicates, the parental strands separate and pair with the new daughter strands. This forces positive superhelical turns into the unreplicated part of the molecule, imposing a stress which, unless relieved, would soon bring replication to a halt. The negative supercoiling catalyzed by DNA gyrase would counteract this stress, allowing replication to proceed. DNA gyrase would act as the required "swivel".

It can also be shown by the use of coumermycin that DNA is actively supercoiled inside E. coli cells. DNA which is converted to the ccc form intracellularly in the presence of the drug stays relaxed, instead of becoming supercoiled as is normal. Thus the supercoiling which is a normal property of ccc-DNA extracted from these cells reflects the activity of DNA gyrase.

Beside replication, certain other reactions involving DNA are also sensitive to its state of supercoiling. For example, the integrative recombination of phage $\lambda$ DNA, both in vivo and in the cell-free model system developed by H. Nash, requires a negatively supercoiled DNA substrate. In fact, it was the study of this reaction that first indicated the presence of a DNA gyrase activity. The reason for the supercoiling requirement in this system is still obscure. RNA synthesis is similarly affected; intracellular transcription is sensitive to coumermycin, only slightly less so than DNA replication, but is resistant in strains with coumermycin-resistant gyrase. As RNA polymerase is known to prefer to bind to supercoiled DNA, the explanation in this case may be simple.

Publications

Gellert, M., Mizuuchi, K., O'Dea, M. H. and Nash, H. A.: DNA gyrase: An enzyme that introduces superhelical turns into DNA. Proc. Nat. Acad. Sci. U.S.A. 73: 3872-3876, 1976.

Gellert, M., O'Dea, M. H., Itoh, T. and Tomizawa, J.: Novobiocin and coumermycin inhibit DNA supercoiling catalyzed by DNA gyrase. Proc. Nat. Acad. Sci. U.S.A. 73: 4474-4478, 1976.

Sequence-specific recombination in bacteria - (Guyer)

A great deal of attention in recent years has been directed to a newly-described class of genetic elements in bacteria termed insertion sequences (IS). IS elements are found in the bacterial chromosome and in a number of plasmids. Their uniqueness stems from the ability of the sequences to transpose themselves and associated gene coding sequences from one replicon to another or from one site on a replicon to a different site. The transposition events are independent of the major homologous recombination system of the host. The ability to participate in genetic rearrangement events such as transposition suggests that IS elements may play a role in the organization of the chromosomal elements of the bacterial cell. IS elements also are involved in gene replication processes.

We have been studying the sequence termed γδ, which is a sequence component of the F plasmid of Escherichia coli. γδ may be an IS element; although not yet fully characterized, it is able to participate in recombination events similar to those which authentic IS elements engage in. The advantage of studying γδ is that the frequency of recombination events associated with it is two to three orders of magnitude higher than the frequency of any other known IS-associated events. The approach being taken is to develop a simple assay for γδ-mediated recombination and then to use that assay to study the genetic requirements and biochemical mechanism of γδ-mediated recombination. This approach would also be applicable to use with authenticated IS elements. The initial aspect of this plan is to construct, by molecular cloning techniques, a suitable plasmid containing γδ. This work is now in progress. In the past year, we have also studied a number of in vivo recombinational phenomena in an attempt to determine the involvement of γδ or other IS elements. Preliminary results were consistent with the participation of γδ in recA-independent chromosome mobilization during conjugation.

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PERIOD COVERED
July 1, 1976 through September 30, 1977

TITLE OF PROJECT (80 characters or less)

Protein Synthesis in Mammals: Mechanisms and Metabolic Controls

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| | Subbaratnam Muthukrishnan | Visiting Associate | LMB NIAMDD |
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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The long range objective of this project is to understand at the molecular level how proteins are synthesized in mammals and how the process of protein synthesis is controlled at the levels of translation and transcription. The topics of current interest are: 1) the isolation and characterization of biologically active messenger RNA from early and term human placentas and from human cancer cells in culture, 2) the translation of eukaryotic mRNA in cell-free systems from wheat germ and Krebs ascites cells, 3) the identification and purification of immunoprecipitable forms of the human placental polypeptide hormones, human placental lactogen (hPL) and human chorionic gonadotropin (hCG) synthesized in cell-free systems, 4) the conversion in cell-free systems of possible precursors of hPL and hCG to mature hormones, 5) the examination of proteins resembling hPL and hCG synthesized in cell-free systems for biological activity.



PHS-6040
(Rev. 10-76)

Project Description

The biosynthesis of the human placental polypeptide hormones, human placental polypeptide hormones, human placental lactogen (hPL) and human chorionic gonadotropin (hCG) in cell-free systems is being studied as one approach toward our object of understanding protein synthesis in mammals. The polypeptide hormones hPL and hCG are synthesized in relatively large amounts in normal placenta and the two hormones appear to be differentially coupled to gestation and placental development. Normally hCG is synthesized only in placental trophoblasts and testes, but it appears ectopically in many cancer cells of human origin. In the latter case the $\alpha$ and $\beta$ subunits of hCG are characteristically excreated in unbalanced quantities. The control of the biosynthesis of hCG seems of particular interest.

Our experimental approach is to isolate biologically active mRNA from early and term placentas and from cells derived from human cancers, to translate these mRNAs in cell-free systems, and to characterize the proteins synthesized which are precipitable by antisera to hPL or hCG.

We have previously shown that poly(A) mRNA from human term placentas directs the synthesis in a cell free system from wheat germ of two immunoprecipitable forms of hPL. The minor species co-electrophoreses on SDS polyacrylamide gels with authentic hPL while the major precursor-like species has an apparent molecular weight of about 3000 greater. This year we have shown that in addition to these two species, very high molecular weight form of hPL ($\bar{M}$ > 150,000) are present in untreated cell-free reaction mixtures, as well as in solubilized immunoprecipitates of both authentic hPL and cell-free products. The high molecular weight forms synthesized in vitro migrate on SDS polyacrylamide gels either with authentic monomeric hPL or with the precursor-like material. Similar high molecular weight forms of hPL have been reported by others in cultured placental slices and in human serum and they may be of biological significance. We have shown that both the precursor-like material and the high molecular weight aggregates synthesized in cell-free systems bind to specific lactogen receptors on liver membranes, thus fulfulling the first requirement for biological activity.

We are presently isolating poly(A) mRNA from early placentas and from HeLa cells known to synthesize the $\alpha$ subunit of hCG and studying the products of their translation in cell free systems. Preliminary data suggests that early placental mRNA directs the synthesis of material with antigenic determinants in common with hCG and that this material has an apparent molecular weight greater than either subunit of the hormone.

We plan to characterize further the products of translation of early placental mRNA and mRNA from HeLa cells and we are working on a unique method for the purification of mRNA which directs the synthesis of hCG.

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PERIOD COVERED

July 1, 1976 through September 30, 1977

TITLE OF PROJECT (80 characters or less)

DNA replication in vitro catalyzed by purified E. coli proteins

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

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SUMMARY OF WORK (200 words or less - underline keywords)

Employee transferred to Cancer Institute. Project will be picked up there as Project Number Z01 CB 8710-02 LMB and will no longer be reported by NIAMDD.



PHS-6040

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PERIOD COVERED
July 1, 1976 to September 30, 1977

TITLE OF PROJECT (80 characters or less)

Structural features of mRNA and their influence on translation

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SUMMARY OF WORK (200 words or less - underline keywords)

The aim of this project is to determine the influence of specific structural features of the 5'-terminal portion of eukaryotic mRNA on initiation of protein synthesis in vitro. By comparing the ribosome-binding efficiencies of mRNA's containing different 5' ends the following conclusions have been reached: 5'-terminal $m^7G$ has a strong promoting influence on mRNA selection by eukaryotic ribosomes. The presence of the 2'-O methyl group as well as the nature of the penultimate residue also have a positive influence at this step, under mRNA excess conditions.



PHS-6040
(Rev. 10-76)

Project Description:

The main objective of this project is to identify the influence of specific structural features of eukaryotic mRNA on their biological activity.

Eukaryotic mRNAs have an unusual structure at the 5' end consisting of a cluster of methylated residues in what is called a 'cap' structure. Using vaccinia virus mRNA and HeLa cell mRNA we are attempting to determine how each portion of this cap structure affects their function by using two approaches; a) specific removal of the $m^7G$ residue by chemical methods and studying the effect of such treatment; b) specific modifications of mRNA's using enzymes purified from vaccinia virus that are involved in modification of 5' ends of mRNA.

The ability of the mRNA to bind to ribosomes and its capacity to stimulate protein synthesis are monitored to follow biological function. Using these assays it was found that removal of the 5'-terminal $m^7G$ results in significant loss of mRNA activity in both the wheat germ and reticulocyte lysate. But enzymatic readdition of the $m^7G$ residue but not of G, results in recovery of activity, stressing the importance of the methyl group at $N^7$ position of guanosine.

Ribosome binding studies under mRNA excess conditions results in an enrichment of mRNA's containing 5'-terminal $m^7GpppA^m$ and $m^7GpppA$ in the ribosome-associated mRNA fraction. Analysis of the bound mRNA under these conditions reveals that the binding efficiencies of mRNAs with different 5'-termini are in the order: $m^7GpppA^m > m^7GpppA > m^7GpppG = m^7GpppG$. It appears therefore that mRNA's containing A caps are more efficiently recognized than those containing G caps. Similar results have been obtained with HeLa mRNA.

A similar approach can be used to determine the influence of other structural features of 5'-termini of mRNA, such as the presence of further 2'-O methylations, $N^6$ methylation and the presence of pyrimidines in the cap structure. These methods could also be used for the study of purified mRNAs coding for specific proteins.

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PERIOD COVERED
July 1, 1976 through September 30, 1977

TITLE OF PROJECT (80 characters or less)

Mammalian Enzymes Involved in Repair or Replication of DNA

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

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COOPERATING UNITS (if any)

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The aim of this project is to identify and characterize enzymes involved in the repair and replication of DNA in higher organisms. Currently we are 1) Surveying eukaryotic sources for a DNA gyrase-like activity, 2) Characterizing a DNA kinase of unusual specificity also from eukaryotic sources, 3) examining the number and characteristics of DNA ligases in rat liver.

Project Description:

Objectives: To isolate and characterize enzymes from mammalian sources which act on DNA, such as DNA kinase and DNA ligase, in order to attempt to understand more fully the processes of DNA repair and replication.

Methods: Standard techniques of biochemistry.

Major Findings: Several aspects of the mechanism of the DNA kinase purified from rat liver nuclei have been examined. The reaction is readily reversible--although the equilibrium lies well in the direction of phosphorylation of DNA termini. Accordingly the termini can be labeled by exchange. A wide variety of nucleotides can participate in the reverse reaction. Kinetic patterns have also been examined; they indicate the absence of a covalent enzyme-substrate intermediate.

In conjunction with Dr. Martin Gellert of this laboratory we are examining rat liver nuclear fractions for DNA gyrase activity. At present we are attempting to distinguish such activity from the similar activity yielded by the nicking-closing enzyme in conjunction with DNA binding proteins, and it is not clear if indeed we are measuring real gyrase activity.

Significance to Bio-medical Research and the Program of the Institute: The enzymes discussed above are thought to function in repair and replication of DNA. Damaged regions in DNA have been implicated in a variety of disease states as well as in aging and differentiation. Enumeration and characterization of the enzymes which carry out repair and replication will hopefully lend insight into the mechanisms of these processes and the aberrations in them which occur in the various diseased states.

Proposed Course: Characterization of the DNA kinase will be continued. Early results indicate the activity to be inhibited by many normally occurring metabolites, and hence readily subject to control. Such control has the potential for controlling DNA degradation and ligation, since the kinase can change the nature of the termini of DNA.

We will continue to purify and test the gyrase-like activity to see if indeed it stems from an active DNA-winding enzyme.

A unique cytoplasmic DNA ligase has been identified in rat liver by Tsukada and Ichimura. We have confirmed their result and are purifying this enzyme in an attempt to see its relation to the other known ligases of this tissue which we have been studying for several years.

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PERIOD COVERED
July 1, 1976 through September 30, 1977

TITLE OF PROJECT (80 characters or less)

Thermodynamics of Hemoglobin S Gelation

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| OTHER: | S. Subramanian | Visiting Associate | LMB | NIAMDD |
| | James Hofrichter | Research Chemist | LCP | NIAMDD |
| | William A. Eaton | Senior Surgeon | LCP | NIAMDD |
| | Allen P. Minton | Research Chemist | LBC | NIAMDD |

COOPERATING UNITS (if any)

None

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Laboratory of Molecular Biology

SECTION
Physical Chemistry Section

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NIAMDD, NIH, Bethesda, Maryland 20014

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CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The polymerization of hemoglobin S molecules into fibers and alignment of these fibers to form a crystalline phase (gelation) is responsible for the deformation (sickling) of the red blood cell in sickle cell anemia. The interpretation of both kinetic and equilibrium experiments on the gelation of hemoglobin S requires thermodynamic information.



PHS-6040
(Rev. 10-76)

Objectives: To gain a basic physical-chemical understanding of the sickling phenomenon and to interpret the behavior of potentially therapeutic additives in terms of their effects upon the kinetics and thermodynamics of gelation.

Methods: Differential scanning and isothermal calorimetry. Optical birefringence and light scattering. Ultracentrifugation, nuclear magnetic resonance spectroscopy and radioactive tracer studies.

Major Findings: Measurements of the solubilities and heats of gelation (8-42°C) have been interpreted in terms of a two-component, two-phase model corrected for the effects of protein non-ideality. At 37°C, gelation is a wholly entropically driven process and values of the standard state thermodynamic parameters have been estimated to be; $\Delta G^\circ = -3$ kcal mol$^{-1}$, $\Delta H^\circ = 0$ kcal mol$^{-1}$, $\Delta S^\circ = 10$ e.u.

The osmotic pressure and sedimentation equilibrium of hemoglobin solutions are accounted for by a model which treats the solution as a suspension of hard quasispherical particles. Non-ideality, due mainly to the protein excluded volume, makes a large contribution to the thermodynamic activity at the hemoglobin S concentrations at which gelation takes place. The tabulated activity coefficients resulting from this analysis will be needed for the proper interpretation of the kinetics of gelation and of the solubility behavior of hemoglobin mixtures.

The concentration dependence of the viscosity of hemoglobin molecules in neutral salt solution has been accounted for by relations describing the viscosity behavior of suspensions of uncharged macroscopic spheres. This result is consistent with the above view of the hemoglobin solution as consisting of hard quasispherical particles.

A systematic study of the effects of ions and organic molecules upon the solubility of deoxyhemoglobin S has been carried out. From these results and the current understanding of the structure of the HbS polymer, we have proposed a stereochemical mechanism of the noncovalent inhibition of gelation of deoxyhemoglobin S. This model accounts for the effects of all inhibitors reported to date and suggests chemical modifications that will be required to achieve more effective inhibition.

Significance: The thermodynamic results and analysis provide the necessary basis for the interpretation of kinetic and equilibrium studies of the gelation of deoxyhemoglobin S.

The results of our solubility studies point to a rational method of discovering more effective inhibitors of the gelation of hemoglobin S.

Proposed Course of Project: Solubility studies aimed at finding more effective inhibitors will be continued. Attempts to verify the proposed

mechanism of non-covalent inhibition of gelation will be pursued by spectroscopic and X-ray crystallographic methods.

Publications:

Ross, P.D., Hofrichter, J. and Eaton, W.A.: Thermodynamics of Gelation of Sickle Cell Deoxyhemoglobin. J. Mol. Biol., in the press, 1977.

Ross, P.D. and Minton, A.P.: Analysis of Non-Ideal Behavior in Concentrated Hemoglobin Solutions. J. Mol. Biol., in the press, 1977.

Ross, P.D. and Minton, A.P.: Hard Quasispherical Model for the Viscosity of Hemoglobin Solutions. Biochem. Biophys. Res. Comm., in press, 1977.

| SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space) | U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT** | PROJECT NUMBER Z01 AM 33054-02 LMB |
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PERIOD COVERED
July 1, 1976 through September 30, 1977

TITLE OF PROJECT (80 characters or less)

Structural Studies on Polynucleotides

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

| | | | |
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| PI: | S. B. Zimmerman | Research Chemist | LMB NIAMDD |
| OTHER: | D. R. Davies | Research Chemist | LMB NIAMDD |
| | M. A. Navia | Staff Fellow | LMB NIAMDD |

COOPERATING UNITS (if any)

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The aim of this project is to obtain structural information on polynucleotides and to provide insights into the factors which control structure, principally through X-ray fiber diffraction and model building studies. Current areas include 1) the structure of the poly U-spermine complex, 2) the structure of a "denatured" polynucleotide, poly A in denaturing solvents, 3) Prediction of structural parameters from observed helical parameters.



PHS-6040
(Rev. 10-76)

Objectives: Obtain structural information on polynucleotides and to provide insights into the factors which control structure.

Methods: Standard techniques of X-ray diffraction and biochemistry; computerized molecular model building.

Major Findings: The complexes of poly U with a variety of polyamines have been examined both chemically for their stoichiometry of interaction and by X-ray diffraction. All the polyamines form the same structure with poly U, but in moreorless well ordered form, and have ca. 1 N/1 P. The polyamines tested were spermine, spermidine, cadavarine, putrescine, ethylene diamine and propylene diamine.

In collaboration with Drs. Navia and Davies, we have evidence for a novel structural form of a polynucleotide. This new ordered structure has been observed in fibers of poly(rA) made from strongly denaturing solvents such as formamide or dimethylsulfoxide. The Xray diffraction pattern together with other evidence suggests that this unique "denatured" structure is a single stranded helix where the adenylylresidues are related by a 180° rotation and a 2.9 Å advance along the helix axis. The backbone is located in the center of the structure and the bases point outwards. The bases do not participate in either direct hydrogenbonding or in stacking interactions.

Significance to Bio-Medical Research and the Program of the Institute: Polynucleotides are crucial in controlling cells in both normal and diseased states. Understanding of polynucleotide structure will hopefully be of aid in understanding and interacting with cells in such diseased states.

Proposed Course: Detailed model building and chemical studies are in progress to determine the structure and stoichiometry of the poly U-spermine complex. The limits to the geometrical approach to structure prediction are being systematically examined.

Publications:

Zimmerman, S. B.: Polynucleotide Models. I. Correlation between Helical Parameters and the Radial Position of the Backbone. Biopolymers 15: 1015-1018, 1976.

Steinert, P. M. Idler, W. W. and Zimmerman, S. B.: Self-assembly of Bovine Epidermal Keratin Filaments in vitro. J. Mol. Biol. 108: 547-567, 1976.

Zimmerman, S. B.: Polynucleotide Models. II. Prediction of the Radial Position and Tilt of the Bases from the Helical Parameters. Biopolymers 16: 749-763, 1977.

| SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space) | U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT** | PROJECT NUMBER Z01 AM 33055-02 LMB |
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PERIOD COVERED

July 1, 1976 to September 30, 1977

TITLE OF PROJECT (80 characters or less)

Thermodynamics of Ligand Binding to Dehydrogenases

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

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COOPERATING UNITS (if any)

NONE

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The similarity of the three-dimensional structure in the NAD-binding domain within the family of dehydrogenases is a familiar concept. An attempt has been made to correlate the structure-function relationship to the thermodynamics of coenzyme binding to several dehydrogenases. The $\Delta G^\circ$, $\Delta H^\circ$ and $\Delta S^\circ$ parameters are broadly similar for the binding of NAD(H) to yeast alcohol dehydrogenase, beef heart and rabbit muscle lactic dehydrogenases and pig heart malate dehydrogenase differing only within a range of 2 Kcal/mol in $\Delta H^\circ$ and compensated by corresponding entropy changes resulting in minimal differences in $\Delta G^\circ$. However, in the case of horse liver alcohol dehydrogenase the $\Delta H^\circ$ values of binding of NAD and NADH are close to zero, the contribution to the binding coming solely from entropic changes. This is suggestive of conformational changes in the enzyme upon binding NAD(H). This view is further confirmed by the thermodynamic parameters of ADP-Ribose binding to the enzymes wherein the abnormality with respect to liver alcohol dehydrogenase vanishes. This study confirms the conclusions about the commonality of the topography of the NAD-binding domain in several dehydrogenases, and establishes that thermodynamics can reflect structure-function correlation with respect to ligand binding.

Project Description:

Objectives: (1) To obtain thermodynamic parameters for the binding of NAD, NADH, ADP-Ribose and Iodosalicylate to several dehydrogenases. (2) To examine the thermodynamic data in relation to structural and functional aspects of the enzymes and attempt to interpret the molecular nature of enzyme-coenzyme, enzyme-inhibitor interactions.

Methods: Batch and flow microcalorimetry.

Major Findings: (1) Thermodynamic data for the binding of NAD, NADH, ADP-Ribose and 5-iodosalicylate to alcohol dehydrogenase (yeast) lactic dehydrogenase (beef heart and rabbit muscle) and malate dehydrogenase (pig heart mitochondrial) are very similar (with only minor variations) for each ligand binding to the various dehydrogenases. This provides some validity to the concept of the similarity of the NAD-binding domain of dehydrogenases deduced from the three-dimensional structure. (2) In contrast, the $\Delta H^\circ$ and $\Delta S^\circ$ of binding NAD and NADH to horse liver alcohol dehydrogenase are strikingly different from those for the other dehydrogenases. This is indicative of conformational changes in liver alcohol dehydrogenase upon coenzyme binding as evidenced by X-ray diffraction studies. (3) ADP-ribose and 5-iodosalicylate do not seem to induce conformational changes while binding in the same domain. The thermodynamic parameters for binding of ADP-ribose and 5-iodosalicylate to liver alcohol dehydrogenase are similar compared to those of other dehydrogenases for the binding of these two ligands.

Significance to Biomedical Research and the Program of the Institute.
This project is designed to obtain a better understanding of the relationship of structural similarities to function in a class of dehydrogenases by studying the thermodynamics of enzyme-ligand interactions using the method of microcalorimetry. The basic knowledge gained therby could pave the way for unraveling some mechanistic aspects of glycolytic and metabolic enzymes.

Proposed Course: (1) It is proposed to extend the study to investigation of kinases and other nucleotide-requiring enzymes. (2) The liver alcohol dehydrogenase is to be studied in greater detail in view of the complexities of proton release upon NAD binding and its importance in the catalysis.

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PERIOD COVERED

July 1, 1976 to June 20, 1977

TITLE OF PROJECT (80 characters or less)

Chromatin Structure and Function

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

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| OTHER: | R. D. Camerini-Otero | Research Associate | LMB NIAMDD |
| | M. Goldsmith | Guest Worker | LMB NIAMDD |
| | W. Melchior, Jr. | Staff Fellow | LMB NIAMDD |
| | J. McGhee | Guest Worker | LMB NIAMDD |
| | G. Sandeen | Chemist | LMB NIAMDD |
| | R. Simon | Research Associate | LMB NIAMDD |
| | B. Sollner-Webb | Staff Fellow | LMB NIAMDD |
| | L. Tack | Chemist | LMB NIAMDD |
| | P. Williamson | Staff Fellow | LMB NIAMDD |
| | M. Zasloff | Research Associate | LMB NIAMDD |

COOPERATING UNITS (if any)

None

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Laboratory of Molecular Biology

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NIAMDD, NIH, Bethesda, Maryland 20014

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Chromatin is the DNA-protein complex present in the nucleus of higher organisms. During the past year, studies of chromatin structure have continued. Further evidence was obtained that the arginine-rich histones, H3 and H4, play a central role in the organization of chromatin subunit (nucleosome) structure. The organization of the DNA within the nucleosome has been studied; the pattern of discrete DNA fragments generated by nuclease attack has been shown to reflect a common set of recognition sites for all nucleases so far studied, spaced at 10 nucleotide intervals along the nucleosomal DNA.

The structure of transcriptionally active regions of chromatin is also under investigation. Nuclease and protease probes have been used to study chromatin structure in globin genes of avian reticulocytes. The organization of the DNA in these genes appears to involve nucleosomes with a structure similar to that present in inactive chromatin. Studies of transcription in model systems suggest that nucleosomes do not necessarily impede the progress of RNA polymera It is likely that the transcription mechanism in eukaryotes involves sliding, exchange or unfolding of nucleosomes.

Project Description:

Objectives: The object of this project is to determine the structure of chromatin and the relationship of this structure to its biological role in cell differentiation.

Methods Employed: Spectrophotometry, analytical ultracentrifugation, dialysis equilibrium, and other standard techniques are used to study the binding of charged molecules to DNA, and the composition of the regions of DNA to which these molecules are bound. Standard chromatographic and electrophoretic techniques are used in purification and identification of histones. RNA polymerase is purified by classical enzymological techniques and its activity examined by membrane filtration, sucrose gradient sedimentation, or radioisotope incorporation. DNA-RNA hybridization methods are used to detech presence of specific polynucleotide sequences. Gel electrophoresis methods are used for measuring DNA and protein size, for examining nucleoprotein preparations, and for detecting chemically cross-linked proteins within chromatin.

Major Findings: DNase cleavage sites in chromatin - We have continued our study of the origin of the regular pattern of DNA fragments generated by nuclease attack on the basic structural subunit of chromatin, the nucleosome. In the case of staphylococcal nuclease, double-stranded fragments are produced that are approximate multiples of 10 base pairs in length, ranging from 40 to about 140 base pairs. In the case of pancreatic DNase (DNase I) and spleen acid DNase (DNase II), the double-stranded fragments do not appear as discrete bands on electrophoretic gels, but a regular pattern of 10.n nucleotide bands (n integer) is generated upon denaturation. We have studied the double-stranded digestion products of DNase I, and shown that they consist of duplex molecules with single-stand "tails" at each end. We have measured the size of these tails by repairing the single-stranded regions with E. coli DNA polymerase II. We deduce that the pattern of cuts on double-stranded nucleosomal DNA is such that cuts on opposite strands are staggered by 8 or 2 nucleotides. Similar experiments with DNase II reveal a stagger of 6 or 4 nucleotides. By the use of 5' terminal labelling of nucleosome DNA in the nucleoprotein monomer, we are able to show that though the cuts produced by the two enzymes occur in different places on the DNA, the cuts all share a common dyad axis. Our results show that the cutting pattern of staphlococcal nuclease is different from that of the other enzymes, but again with the same dyad axis. The simplest explanation of this result is that although the enzymes cut at different characteristic sites, they bind to a common site which occurs once in every ten nucleosides on each DNA strand of the nucleosome.

Arginine-rich histones and DNA folding - We had shown earlier that the arginine-rich histones, H3 and H4, play a central role in the organization of the nucleosome. Using a variety of enzymic probes, we showed that this histone pair is necessary and sufficient, when complexed with DNA, to generate a structure with nucleosome-like properties. We have now extended this study to include the effects of the H3/H4 pair on DNA supercoiling, and have shown

that H3/H4 is able to induce formation of supercoil in closed circular DNA. No single histone species, or mixture lacking H3 or H4, is effective, but the H3/H4 pair is as efficient, on a weight basis, as a mixture of all four "core" histones, H2A, H2B, H3 and H4. We have also shown that a histone octamer containing four H3 and four H4 molecules is able to fold a DNA fragment of the size found in nucleosome monomers into a compact structure like a nucleosome. In the course of these studies, we have estimated the approximate energy required to bend DNA into a compact structure the size of a nucleosome, using the known bending force constant of DNA. We have also calculated the free energy associated with formation of histone-histone contacts during a hypothetical folding and unfolding reaction of nucleosomes. The calculations show that only one or two new histone-histone contacts would be enough to stabilize the folded structure, and suggests that an unfolding reaction of the nucleosome could involve a delicate balance of forces.

Transcription of globin genes in vitro from chromatin - The use of mercury-labelled nucleoside triphosphates to study in vitro transcription of chromatin has become widespread, since it seemed straightforward to separate newly synthesized RNA from endogenous message using thiol-agarose columns. We have discovered that the usual assay methods can give rise to large false positive results, because E. coli RNA polymerase also uses endogenous RNA as a template. The duplex formed from this reaction, containing that RNA and a complementary, Hg-labelled strand, is trapped on thiol-agarose, and the endogenous message is counted as though it were newly synthesized. We have developed new assay techniques using mercury-labelled precursors which we hope will eliminate this problem.

Active gene structure - We continue our investigation of the organization of proteins in the neighborhood of actively transcribed genes, in particular the adult globin genes of avian red blood cells. We have had evidence for some years that such genes, when isolated from reticulocytes of anemic adult ducks, are covered with protein. More recently, workers in several laboratories have shown that the nucleosome monomer fraction from such cells contains the genomic abundance of globin sequences, showing that these genes are all packaged in a structure with the repeat distance of a nucleosome. To demonstrate that this structure is indeed a nucleosome, we nave examined the globin gene distribution in the subnucleosome fragment pattern produced by nuclease digestion. Our results show that the internal organization of globin genes in cells capable of globin RNA synthesis is that of a nucleosome.

These results suggest that polymerase molecules must be capable of passing through regions of chromatin that are histone-covered. We have studied a model "chromatin" template made by reconstitution of the four core histones onto DNA from bacteriophage T7. The transcription of T7 DNA by E. coli polymerase has been well studied. When we use this polymerase to transcribe the T7-histone complex, we find that in 0.45 M NaCl, where histones are still attached to DNA, the rate of chain elongation is not reduced relative to that on naked DNA by the presence of histones.

Thus, there is no reason to suppose that histones bound to DNA provide an insurmountable obstacle to passage of polymerase. The system provides a

method for studying the mechanism involved in this passage, which may require sliding, exchange, or unfolding of the histone complex.

Significance to Bio-medical Research and the Program of the Institute: Chromatin is the nucleoprotein complex that appears to contain the chemical information for cellular differentiation. Understanding of the organization of chromatin proteins on DNA and the way in which they affect transcription and other DNA functions in higher organisms will lead to an understanding of how cells differentiate, and how they maintain this differentiated state, in which only a relatively small fraction of the total gene population of the cell is expressed. This is a central problem in cell biology.

Proposed Course of Project: We will continue to study the organization of nucleoprotein in the neighborhood of the globin gene of avian reticulocytes, and other active genes. We are also continuing studies of nucleosome structure using homogeneous, sequenced DNA segments in the nucleohistone complexes.

Publications:

Felsenfeld, G., Camerini-Otero, R.D. and Sollner-Webb, B.: The Structure of Chromatin and Its Reconstruction. In Nierlich, D.P. and Rutter, W. (eds.): Molecular Mechanisms in the Control of Gene Expression, California, W. A. Benjamin, 1976, pp. 1-6.

Felsenfeld, G., Sollner-Webb, B., Camerini-Otero, R.D. and Melchior, W. Jr.: Organization of Proteins in Chromatin. In Papaconstantinou, John (ed.): 34th Symposium of the Society for Developmental Biology, The Molecular Biology of Hormone Action, 1976, pp. 3-13.

Camerini-Otero, R.D., Sollner-Webb, B. and Felsenfeld, G.: The Organization of Histones and DNA in Chromatin: Evidence for an Arginine-Rich Histone Kernel. Cell 8: 333-347, 1976.

Sollner-Webb, B., Camerini-Otero, R.D. and Felsenfeld, G.: Chromatin Structure as Probed by Nucleases and Proteases: Evidence for the Central Role of Histones H3 and H4. Cell 9: 179-193, 1976.

Sollner-Webb, B. and Felsenfeld, G.: Pancreatic DNAase Cleavage Sites in Nuclei. Cell 10: 537-547, 1977.

Zasloff, M. and Felsenfeld, G.: Use of Mercury-Substituted Ribonucleoside Triphosphates Can Lead to Artefacts in the Analysis of In Vitro Chromatin Transcripts. Biochem. and Biophys. Res. Comm. 75: 598-603, 1977.

Camerini-Otero, R.D. and Felsenfeld, G.: Supercoiling energy and nucleosome formation: the role of the arginine-rich histone kernel. Nucl. Acids Res. 4: 1159-1182, 1977.

Zasloff, M. and Felsenfeld, G.: Analysis of in vitro Transcription of Duck Reticulocyte Chromatin Using Mercury-Substituted Riconucleoside Triphosphates. Biochem., in press.

Camerini-Otero, R.D., Sollner-Webb, B., Simon, R. H., Williamson, P., Zasloff, M. and Felsenfeld, G.: Nucleosome Structure, DNA Folding and Gene Activity. Cold Spring Harbor Symp. Quant. Biol., in press.

| SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space) | U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT | PROJECT NUMBER Z01 AM 34002-13 LMB |
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PERIOD COVERED

July 1, 1976 to September 30, 1977

TITLE OF PROJECT (80 characters or less)

Proteolytic Enzymes

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

| | | | |
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| OTHER: | E. Subramanian | Visiting Associate | LMB NIAMDD |
| | G. Cohen | Research Chemist | LMB NIAMDD |
| | E. Silverton | Research Chemist | LMB NIAMDD |
| | M. Liu | Visiting Associate | LMB NIAMDD |

COOPERATING UNITS (if any)

None

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Laboratory of Molecular Biology

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INSTITUTE AND LOCATION

NIAMDD, NIH, Bethesda, Maryland 20014

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| 3 | 3 | 0 |

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The refinement of γ-chymotrypsin has continued by real-space electron density fitting using both automated computer procedures and computer graphics. Additional refinement using differential fourier synthesis combined with model building using soft constraints is underway. Comparison with other known serine proteases is planned.

A 2.5 Å map of the acid protease from Rhizopus Chinensis has been calculated. The backbone of the entire molecule can be traced. Although the sequence data remain incomplete, a majority of amino acid assignments can be deduced with confidence based on sequence homology with other acid proteases and the quality of the map. A molecular model is under construction by means of computer graphics. Refinement methods developed for use with γ-chymotrypsin should be useful in improving the model using 2.0 A data that are already available.



FORM 2040
(Rev. 10-76)

Project Description:

Objectives: The determination of the three-dimensional structure of proteolytic enzymes, in particular, the structure of γ-chymotrypsin and of the acid protease from Rhizopus chinensis.

Methods Employed: X-ray diffraction analysis of single crystals including heavy atom isomorphous replacement, model building, coordinate refinement in both real and reciprocal space, and computer graphics.

Major Findings: 1. The refinement of native γ-chymotrypsin at 1.9 Å in progress is now being done by differential synthesis combined with model building using both computer graphics and non-interactive computer programs. The potential of using other crystallographic methods, such as least squares with restraints, is being considered. Experience gained in the application of these refinement methods should prove valuable in the refinement of other proteins underway in this section.

2. All α-carbon positions for the acid protease have been assigned based on a 2.5 Å map. Most of the sequence has also been placed and the resultant 3-dimensional molecular model is under construction on a computer graphics system.

Proposed Course: To refine the model constructed from the 2.5 Å map of the acid protease using the available 2.3 Å data with methods tested on γ-chymotrypsin.

Significance to Bio-medical Research and the Program of the Institute:

Although the general mechanism of action of the serine proteases is fairly well understood, there is still some disagreement concerning the precise details. The refinement of γ-chymotrypsin at high resolution and different pH's should help resolve the problem.

The acid protease is one of a large class of enzymes that include pepsin, rennin and the cathepsins. It has sequence homology with other members of the class and the elucidation of its structure should ultimately reveal the mechanism of action of these proteins.

Publications:

Subramanian, E., Swan, I.D.A., Liu, M., Davies, D.R., Jenkins, J.A., Tickle, I.J. and Blundell, T.L.: Homology among acid proteases: Comparison of crystal structures at 3 Å resolution of acid proteases from Rhizopus chinensis and Endothia parasitica (x-ray diffraction/inhibitor binding/enzyme cleft). Proc. Natl. Acad. Sci. U.S.A. 74: 556-559, 1977.

Subramanian, E., Liu, M., Swan, I.D.A. and Davies, D. R.: The crystal structure of an acid protease from Rhizopus Chinensis at 2.5Å resolution. In Tang, Jordan and Hartsuck, Jean (eds): Acid Proteases: Structure, Function, and Biology. Plenum Publishing Corp., New York, N.Y. pp. 000-000 (in press).

| SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space) | U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT** | PROJECT NUMBER Z01 AM 34003-09 LMB |
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PERIOD COVERED

July 1, 1976 to September 30, 1977

TITLE OF PROJECT (80 characters or less)

Crystal Structure Investigation of Antigen Antibody Interaction

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

| | | | |
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| | Y. Satow | Visiting Fellow | LMB NIAMDD |

COOPERATING UNITS (if any)

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| M. Potter | Biologist | C LCBGY |
| S. Rudikoff | Microbiologist | C LCB |
| N. Glaudemans | Chief, Sec on Carbohydrates, LC, NIAMDD | |

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

A backbone model for the low resolution study of the human IgG1 immunoglobulin "Dob"" has been proposed.

Improvement of the phase determination for the M603 Fab is under way by means of density modification with solvent constraints on the 3.1Å map. Refinement of the atomic coordinates is contemplated.

The immunoglobulin J539 Fab fragment has yielded a poor quality map at 4.5Å. Attempts are under way to solve the structure in a manner similar to that used for "Dob".

The binding sites of M315, J539 and E109 have been built from sequence data and analogy with the M603 molecule. The models are being correlated with binding studies (M315 & J539) and with serological studies (E109).

Sequence variations in immunoglobulins are being correlated with the known three-dimensional structures. These correlations could be useful in locating specificity determining residues in immunoglobulins of unknown structure.

Project Description:

Objectives: A comparative investigation of the crystal structure of the Fab fragment of immunoglobulin molecules that bind strongly to several different antigens. In this way, it is hoped to map out the binding site of antibodies and to illustrate the mechanism of antigen-antibody interaction.

Methods Employed: Preparation and analysis of pure materials. Crystallization and x-ray diffraction analysis with: a) heavy atom isomorphous replacement, b) rotation function, c) molecular replacement in real space. Model building. Computer comparison of structures. Computer automated refinement of phases and atomic coordinates.

Major Findings: 1. Using the previously determined domain structures from M603 and other published immunoglobulin fragments, it has been possible to interpret a 6 Å map of "Dob" by means of a computer search program. The quateruary structure of the molecule determined is in agreement with our earlier published map interpretation and provides a solution, as well, for the carbohydrate moiety of the molecule. The angle between the variable and constant regions of the Fab is intermediate between the angles found for other Fabs and emphasizes the flexibility of the switch region of the molecule. The method developed appears to be of general applicability.

2. From the model of the antigen binding site of M315, a ligand binding configuration has been proposed which can account for the results of published ligand binding studies in solution. This model has since served as the starting point for further ligand binding studies by other investigators using more sensitive techniques. Sequence variations in mouse inulin binding proteins and the results of serological studies of these proteins correlate very well with the model of the variable region which we have built for one of them (E109).

Proposed Course: 1. The M603 structure will be refined using real and reciprocal space methods that have proven effective on other proteins.

2. The investigation of J539 will be continued using a real space molecular replacement procedure as was done with "Dob" as well as other molecular replacement techniques.

3. Attempts will be continued to obtain crystals of other mouse myeloma proteins and rabbit antibodies.

Significance to Bio-medical Research and the Program of the Institute:

An understanding of the molecular basis of interaction between antibody and antigen is fundamental to our understanding of the immune response.

Publications:

Padlan, E.A., Davies, D.R., Pecht, I., Givol, D. and Wright, C.: Model-

building Studies of Antigen-binding Sites: The Hapten-binding Site of MOPC-315. Cold Spr. Harb. Symp. of Quant. Biol., Vol. XLI, 627-637, 1977.

Padlan, E.A., Davies, D.R., Rudikoff, S. and Potter, M.: Structural Basis for the Specificity of Phosphorylcholine-binding Immunoglobulins. Immunochemistry 13, 945-949, 1976.

Padlan, E.A.: Structural Basis for the Specificity of Antibody-antigen Reactions and Structural Mechanisms for the Diversification of Antigen-binding Specificities. Quarterly Review of Biophysics, 10, 35, 1977.

Padlan, E.A.: Structural Implication of Sequence Variations in Immunoglobulins. Proc. Nat. Acad. Sci. USA (in press).

| SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space) | U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT** | PROJECT NUMBER Z01 AM 35000-13 LMB |
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PERIOD COVERED
July 1, 1976 to September 30, 1977

TITLE OF PROJECT (80 characters or less)
Chemical and Structural Investigations of Nucleic Acids and Related Substances

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

| | | | | |
|---|---|---|---|---|
| PI: | H. Todd Miles | Chief, Sec. on Org. Chem. | LMB | NIAMDD |
| OTHER: | Frank B. Howard | Research Scientist | LMB | NIAMDD |
| | Girjesh Govil | Visiting Scientist | LCP | NIAMDD |
| | Hansie Mathelier | Visiting Fellow | LMB | NIAMDD |
| | Masako Muraoka | Visiting Fellow | LMB | NIAMDD |

COOPERATING UNITS (if any)
E. D. Becker, Laboratory of Chemical Physics, NIAMDD

LAB/BRANCH
Laboratory of Molecular Biology

SECTION
Organic Chemistry Section

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20014

| TOTAL MANYEARS: | PROFESSIONAL: | OTHER: |
|---|---|---|
| 6 | 4 | 2 |

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

This project has the long range objective of studying the chemistry and structure of nucleotides, polynucleotides and nucleic acids and the application of these studies to the understanding of biochemical and biological processes. Topics of current interest include: (1) structure and reactivity of polynucleotides and the relation between the two; (2) stabilization of ordered forms of nucleic acids and polynucleotides; (3) spectroscopic studies of polynucleotides: infrared, ultraviolet, and circular dichroism; (4) design and synthesis of new polynucleotides having properties required for the foregoing studies.



PHS-6040
(Rev. 10-76)

Project Description:

A·I and A·G Polynucleotide Pairing: Controlling Effect of NH Hydrogen Bonds to Solvent Water. A major objective of this project has been determination of the ways in which hydrogen bonding of nucleic acid bases affects the specificity and stability of polynucleotide interaction. In the past, we have examined direct effects in which hydrogen bonding is enhanced by addition of amino groups in appropriate positions or by direct blocking with bulky alkyl residues.

In the present study we have investigated an indirect effect of hydrogen bonding, which is mediated by the energetics of the helix-coil equilibrium. A·I pairs are known to form, but A·G pairs have not been observed. There have been contradictory predictions that such pairs will form (Rich) and that they will not (Crick). An acceptable pair of hydrogen bonds can be formed between A and G, but one proton of the G amino group would then not be able to make a hydrogen bond to solvent water because of steric exclusion of water by proximity of 2-CH of A. While lack of this hydrogen bond would clearly be destabilizing to the helix, less complete stacking of the coil would presumably favor the helix. Since present knowledge of the energetics of the equilibrium does not permit a reliable prediction, an experimental approach to the problem was necessary.

The problem of A, G interaction cannot be usefully examined by mixing poly(A) with poly(G) because of the highly stable self-structure of G. We have investigated the same general principle, however, by observing the interaction of poly(I) with poly 2-aminoadenylic acid (poly($2NH_2A$)), in which the 2-substituents of A and G are transposed. Neither homopolymer in this case has a self-structure sufficiently stable to block hetero interaction. The prediction is that if inability of an NH proton to hydrogen bond to water in the helix can prevent helix formation, poly(I) and poly($2NH_2A$) will form a 1:1 complex (or possibly not interact at all). If the H-bond to water cannot play such a role, however, the two polymers should form an $AI_2$ triple helix.

Experiments showed that a stable helix is formed, and detailed examination of the stoichiometry demonstrated that a 1:1 complex was formed and that a 1:2 complex was not. Assurance that the observed blocking of A·I interaction at the pyrimidine (N1) bonding site of A did not result from direct steric blocking of I by the 2-amino group was provided by studies with poly 2-methyladenylic acid (poly(2MeA)). The $CH_3$ group is slightly larger than $NH_2$ but is not a hydrogen bond donor. If the $CH_3$ (or the $NH_2$) group is large enough to block pairing, then poly(I) would form only a 1:1 complex and not a triple helix. In this case, experiments demonstrated that only a 1:2 $AI_2$ complex is formed. We conclude that inability to form a hydrogen bond to water can control the direction of the equilibrium and prevent helix formation. An A·G pair is therefore not expected to form.

The "alternative pairing" proposed for DNA by Donohue and Trueblood in 1960 (G bonded to N7 of A) is likewise ruled out since the same kind of steric

exclusion of water in the helix exists here. This conclusion was confirmed experimentally by synthesizing and examining poly($8NH_2A$). A complex of low stability was formed with poly(I), bonded at N1 of A and not at N7.

In the course of this work, it was also necessary to reinvestigate interaction of poly(A) with poly(I) and show that a prior report of a 1:1 complex of these polymers was erroneous. Only a triple helix is formed.

These experiments also led to a surprising conclusion about relative stabilities of N1 and N7 bonding sites in the $AI_2$ helix. Bonding at N7 is markedly more stable than at N1 in contrast to the A, U system where the opposite is true. These observations are of interest in connection with the wobble A·I pairing of mRNA to the anticodon (Howard, Hattori, Frazier and Miles).

New Methods of Spectroscopic Analyses of Polynucleotide Combining Ratios. The central conclusions of the foregoing study depend directly upon a demonstration of stoichiometry of those complexes which are formed and, equally, upon clear evidence that other chemically plausible complexes are not formed. Though the usual methods of measuring mixing curves may be adequate to establish the combining ratio of complexes which do exist, they have frequently led to erroneous conclusions and have been unable to show that a hypothetical complex is not formed. There has always been the possibility when mixing curves failed to show formation of a particular complex that there might exist a different wavelength capable of demonstrating the complex. Even when a complex can be shown to exist, the spectroscopic detectability is often so small that a more favorable wavelength is desirable.

We have recently developed new methods of analysing spectroscopic data which eliminate most of the ambiguity and uncertainty frequently associated with studies of stoichiometry in the past. The first method employs the ultraviolet spectra (200-400 nm) of mixtures of polymers and the second their derivative spectra, $d\varepsilon/d\lambda$ vs $\lambda$, both collected and processed by a digital computer (Howard et al., 1976). Angles of intersection of the mixing curves are defined as explicit dependent variables $\theta_1$ and $\theta_2$ and calculated by the computer as a continuous function of wavelength.[2] Maxima and minima in these dispersion curves correspond to optimum wavelengths for detecting a complex of a particular composition. Conversely, when $\theta i = 180°$ over the entire spectrum, there is no break in the mixing curve at any wavelength, and we conclude that a complex corresponding to mole fraction Xi is not formed.

A further method of analysis uses the derivative spectra, $d\varepsilon/d\lambda$ vs $\lambda$, of polynucleotide mixtures. The ultraviolet spectra are measured and transmitted to an on-line computer, which differentiates the spectra, and computes the angles of intersection of the mixing curves as continuous functions of wavelength. We have found that the derivative spectra themselves are quantitatively reproducible, highly characteristic, and often show greater

changes on complex formation than the original ultraviolet spectrum. In many cases the derivative mixing curves are more sensitive to formation of different complexes, and in the majority of cases the most favorable wavelengths are those at which the ultraviolet mixing curves cannot detect any interaction at all (that is, wavelengths at which $\theta i$ is approximately 180°). By calculating the wavelength dispersions of $\theta i$ and using derivative mixing curves we can obtain unambiguous evidence not only that a particular complex is formed, but also that a plausible complex is not formed (Howard and Miles).

Structure of Poly(8BrA), an All-Syn Polymer. In order to study the effects of the unusual syn conformation on polynucleotide structure, we had previously synthesized the first polynucleotide in which all of the base residues were constrained to be syn (glycosidic angle $\chi \sim 240°$) by bulky Br residues. The polymer was shown to exist as a highly stable double helix with extensive base stacking. In order to obtain more detailed information of the structure, the study was continued with a combination of theoretical and experimental methods in collaboration with Dr. Girjesh Govil (LCP; permanent address: Tata Institute for Fundamental Research). Potential energy calculations (CPF) by Dr. Govil produced a set of possible structures having relatively low non-bonded interaction energy. These candidate structures were then examined for consistency with experimental evidence (NMR, IR, UV) and for suitability as components of a hydrogen-bonded double helix. Many of the low energy structures would have placed the phosphates inside the helix and bases outside and were thus unsuitable for electrostatic and hydrogen bonding reasons. Others had C2 endo sugar conformations, excluded by NMR evidence. A single structure survived the exclusion process. Further refinement of this structure showed that only slight changes were required to obtain a very satisfactory double helix having parallel strands, stacked bases and a 2-fold rotation axis parallel to the helix axis. Of three possible hydrogen bonding schemes only a symmetrical one bonded at N1 and an amino proton is capable of satisfying the geometrical requirements. Each strand has C3 endo geometry and $\phi' = 216°$, $\omega' = 280°$, $\omega = 294°$, $\phi = 179°$, $\chi = 243°$, and $\psi = 57°$. Such a conformation leads to approximately 8 nucleotide units per turn of the helix and an axial rise of 3.9 Å.

One of the most interesting aspects of the results obtained in this study is that although the orientation of the base and the hydrogen-bonding scheme are different in the cases of poly(A) and poly(8BrA), their backbone conformations are very similar, both in the ordered and random-coil forms. These results provide further evidence that (a) the basic nucleotide structure is stabilised by short-range interactions within the backbone itself, (b) intra- and inter-strand base-base interactions (stacking and hydrogen bonding) provide additional stabilisation to the structure corresponding to the conformational angles found in ordered RNA, and (c) a major fraction of a randomly coiled single-strand has a conformation similar to that of each strand in a double helix (Govil, Fisk, Howard, and Miles).

Circular Dichroism (CD) of Polynucleotide Helices in Which the Purine "Strand" is Composed of Monomers. Both ultraviolet absorption and CD spectra of polynucleotides above 200 nm are due to electronic transitions of the heterocyclic bases. The magnitude of these extrema is greatly enhanced by base-base interactions, but the question remains whether the observed strong CD signals are significantly influenced by asymmetric centers in the ribose-phosphate chain. Does this backbone merely serve as a scaffolding to provide a chiral geometry for the bases or does it interact with and perturb the electronic transitions of the bases to a significant extent? An experimental approach to the problem is provided by our previous discovery that purine monomers are capable of forming helices with complementary pyrimidine polynucleotides. The monomeric components may be nucleotides, nucleosides, purines, or 9-alkyl purines. In all of these helices there is no continuous ribose-phosphate chain in the purine strand and in those formed with last two classes of monomers there is not even an asymmetric center. The only asymmetry of the purine components is then that induced by incorporation in the helix. We have found that in several triple helices formed by 2-aminoadenine monomers with poly(U) the CD spectra are quite similar to those of the corresponding polymer-polymer helices with respect to wavelength, magnitude, and sign at wavelengths above $\sim$ 240° nm. The unusual negative first extremum observed in the polymer-polymer helices (Howard et al., 1976) was reproduced quite well with 2-aminoadenine and 9-Me-2-aminoadenine complexes, which have no asymmetric centers. This extremum is due to the B2U transition of $2NH_2A$ and has no significant contribution from the poly(U) strand. At lower wavelength ($\sim$ 220 nm) the sign of the strongest extremum is reversed in several cases. The two-stranded complex formed between poly(C) and 5'-GMP was found to have a CD spectrum remarkably similar to that of poly(G)·poly(C) at all wavelengths. Even the intense negative extremum at 211 nm was closely reproduced.

We conclude from these experiments that the CD spectra above 200 nm are determined largely if not entirely by the helical assay of bases, essentially unperturbed by the asymmetric ribose-phosphate backbone (Mathelier and Miles).

Publications:

Howard, F.B., Frazier, J. and Miles, H.T.: Poly(2-aminoadenylic acid): Interaction with Poly(uridylic acid). Biochemistry 15: 3783-3795, 1976.

Howard, F.B., Frazier, J. and Miles, H. Todd: Stable and Metastable Forms of Poly(G). Biopolymers 16: 791-809, 1977.

Govil, G., Fisk, C., Howard, F.B., and Miles, H.T.: Structure of Poly 8-Bromoadenylic Acid: Conformational Studies by CPF Energy Calculations. Nucleic Acids Research, in press.

| SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space) | U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT** | PROJECT NUMBER Z01 AM 35050-06 LMB |
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PERIOD COVERED
July 1, 1976 to September 30, 1977

TITLE OF PROJECT (80 characters or less)

Replication, Recombination and Repair of Microbial DNA

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

| | | | | |
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| OTHER: | Robert Bird | Staff Fellow | LMB | NIAMDD |
| | Tateo Itoh | Visiting Associate | LMB | NIAMDD |
| | Kazuo Yamaguchi | Visiting Associate | LMB | NIAMDD |
| | Haruo Ohmori | Visiting Fellow | LMB | NIAMDD |

COOPERATING UNITS (if any)

None

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Laboratory of Molecular Biology

SECTION
Molecular Genetics Section

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20014

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| 5 | 5 | 0 |

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

In vitro studies on replication of plasmid Col E1 DNA has been the major subject of investigation. The origin of replication was located at a unique site in the nucleotide sequence that we determined. Ribonucleotide(s) was found to attach to a DNA fragment first formed in the process of plasmid replication. The participation of various bacterial functions for the plasmid replication was studied. The gene that determines the resistance to novobiocin was identified as that for DNA gyrase.



PHS-6040
(Rev. 10-76)

Project Description:

Replication of closed-circular colicin E1 plasmid (Col E1) DNA can be initiated and completed in extracts of Escherichia coli. The major products of in vitro replication are completely replicated molecules and a unique type of early replicative intermediate containing a newly synthesised DNA fragment(s) in a small replication loop. The fragment has an average length of approximately one fifteenth of the unit length of the plasmid molecule and has a sedimentation constant of approximately 6 S. Synthesis of the intermediates is inhibited by rifampicin but most of the intermediates can complete replication in the presence of rifampicin. The first synthesis of a DNA fragment is initiated at a specific site on the H strand and depends on the function of E. coli DNA-dependent RNA polymerase. Subsequent synthesis of the DNA fragment on the L strand does not involve the RNA polymerase.

Cleavage maps of colicin E1 plasmid DNA and its smaller derivative, pNT1 DNA, were constructed using restriction endonucleases. The nucleotide sequence of a region which contains the origin of replication was determined. The site of the nucleotide from which DNA replication is initiated was determined with 6S L-fragments, which is the DNA fragment first made on Col E1 DNA. The fragments were labeled with [$\gamma$-$^{32}$P]ATP and polynucleotide kinase at the 5'-OH groups which were uncovered by alkali treatment. The site is one of three consecutive nucleotides, dA, dA, and dC located at a unique position. One or a few rA residues were found to be attached to some of the DNA molecules. The transition from the primer RNA to DNA occurs in a region consisting of a segment of five A residues. Both sides of this segment are very rich in G and C. This is the first case where the origin of DNA replication is determined at the precise location in the nucleotide sequence.

Attachment of RNA to 6S L-fragments was shown by the transfer of $^{32}$P-label in the DNA derived from one of the [$\alpha$-$^{32}$P]dNTPs to one of 2'(3')rNMP after alkali hydrolysis of 6L-fragments. It was found that rA-dA and rA-dC linkages predominate ribonucleotide-deoxyribonucleotide linkages in the 6S L-fragments. This result is in complete agreement with the expectation based on the analysis of the nucleotide sequence of the region that contains the origin of replication.

In vitro Col E1 DNA replication is carried out by bacterial functions. Purification of enzymes that participate in replication and reconstruction of a replication system by combination of purified enzymes are studied. DNA gyrase was found to be one of the essential enzymes and the gene responsible for resistance to coumermycin was identified as a structural gene for the enzyme.

DNA gyrase was shown to be essential for replication of T7 phage DNA in vivo. The first round of replication of the phage DNA which is presumed to occur in a linear structure was inhibited by coumermycin. The replication in the resistant bacteria was not inhibited.

Publications:

Sakakibara, Y., Suzuki, K. and Tomizawa, J.: Formation of Catenated Molecules by Replication of Colicin E1 Plasmid DNA in Cell Extracts. J. Mol. Biol. 108: 569-582, 1976.

Gellert, M., O'Dea, M.H., Itoh, T. and Tomizawa, J.: Novobiocin and coumermycin inhibit DNA supercoiling catalyzed by DNA gyrase. Proc. Natl. Acad. Sci. U.S.A. 73: 4474-4478, 1976.

Tomizawa, J., Ohmori, H. and Bird, R.E.: The Origin of Replication of Colicin E1 Plasmid DNA (cleavage map/nucleotide sequence/polynucleotide kinase/primer). Proc. Natl. Acad. Sci. U.S.A. 74: 1865-1869, 1977.

| SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space) | U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT | PROJECT NUMBER ZO1 AM 36002-03 LMB |
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PERIOD COVERED
July 1, 1976 to September 30, 1977

TITLE OF PROJECT (80 characters or less)

Novel Recombination Systems

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

| | | | |
|---|---|---|---|
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| OTHER: | Mark Guyer | Staff Fellow | NIAMDD:LMB |

COOPERATING UNITS (if any)

NONE

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Microbial Genetics Section

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20014

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| 1-1/12 | 1-1/12 | 0 |

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The Tn9 transposon - a genetic element which carries a gene for chloramphenicol-resistance flanked on both sides by IS1 (an insertion sequence) - possesses two remarkable features. (1) It is able to insert itself as a unit into a variety of unrelated chromosomes and at many different sites. (2) it can generate deletions of portions of itself and/or of adjacent chromosomal DNA. Satisfactory understanding of the genetic parameters of the latter activity has yet to be achieved. Part of the problem is that Tn9 can be so "plastic" that standard genetic crosses frequently do not yield expected progeny.

Project Description:

The Tn9 transposon has a gene for chloramphenicol-resistance (cam) of about 600 base pairs flanked at both ends by IS1, an insertion sequence of about 800 b.p. Tn9 can insert into a variety of unrelated genomes including that of bacteriophage λ. λ carrying Tn9 (called λcam) can lysogenize E. coli and confer upon it resistance to chloramphenicol. However, upon lytic growth of λcam, a few percent of the λ are found to have lost the cam gene. Since the loss of cam has not been observed in the lysogenic state, it is possible that a repressible λ function is involved in the loss of cam. To identify this function, various λ mutations were crossed into λcam. One isolate, λcam red3 (recombination defective) lost cam at a very low rate, implicating the Red function. However, other λcam red strains did lose cam. Closer scrutiny showed that the λcam red3 phage has deletion of about 6% of the λ genome adjacent to Tn9. Other deletions of λ on either side of Tn9 have been found but it is not clear how these deletions influence the rate of loss of cam. The frequency of the deletions and their location adjacent to Tn9 indicates that Tn9 is a deletion-generating element.

A particularly interesting Tn9 promoted deletion of λ has recently been found. Bacteria infected with this λcam segregate chloramphenicol-sensitive cells at a high frequency, are not immune to λ, and do not produce plaque-forming particles. The λcam has been found to be present as an unstable plasmid (not integrated), has a deletion of about 23% of the λ genome, and appears to be missing two important regulatory genes of λ, genes N and cI.

Publications:

Rosner, J. L. and Gottesman, M. M.: Transposition and deletion of Tn9. A transposable element carrying the gene for chloramphenicol resistance. In Bukhari, A., Shapiro, J. and Adhya, S. (Eds.): DNA Insertion Elements, Plasmids, and Episomes. New York, Cold Spring Harbor, 1977 (in press).

| SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space) | U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT | PROJECT NUMBER Z01 AM 36051-09 LMB |
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PERIOD COVERED
July 1, 1976 - September 30, 1977

TITLE OF PROJECT (80 characters or less)

Genetics and structure of the oncogenic virus, SV40

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

| | | | |
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| PI: | Robert G. Martin | Chief, Sec. on Microbial Genetics, | LMB NIAMDD |
| OTHER: | Maria Persico-DiLauro | Visiting Fellow | LMB NIAMDD |
| | Carol A. F. Edwards | Staff Fellow | LMB NIAMDD |
| | Ariella Oppenheim | Visiting Scientist | LMB NIAMDD |

COOPERATING UNITS (if any) David Livingston, Sidney Farber Cancer Institute,
D. G. Tenen, Sidney Farber Cancer Institute,
Chungming Chang, Visiting Fellow, LCBGY NCI
Peter T. Mora, Head, Macromolecular Biol. Sec., LCBGY NCI
~~Jeffrey Anderson, Stanford Univ. Medical Center~~

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SECTION
Microbial Genetics Section

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NIAMDD, LMB, Bethesda, Maryland 20014

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

We have been working on the molecular basis of transformation by Simian virus 40. We have demonstrated that the tumor specific transplantation antigen is related to the tumor antigen. In cells transformed by temperature-sensitive mutants the transformed phenotype is temperature-sensitive. We have examined the distance between origins of DNA synthesis in normal and transformed cells. We have interpreted our results to indicate that the SV40 tumor antigen transforms by acting as a new initiator of DNA synthesis.

Project Description:

Objective: To analyze the molecular basis of transformation by SV40 using conditional lethal mutants.

Major Findings: (a) We have demonstrated that the tumor specific transplantation antigen (TSTA) associated with SV40 transformed and lytically infected cells, copurifies with the tumor antigen (TA). In addition the time course of appearance of TSTA parallels that of TA in lytic infection; and TSTA and TA are present in most tsA mutant-transformed cells, although the antigens disappear coordinately in one rat line. We have postulated that the two antigenic activities represent different antigenic sites of the same polypeptide. (Anderson and Martin associated with C. Chang, P. Mora and D. Livingston).

(b) The distance between origins of DNA replication in normal and SV40 transformed cells have been analyzed. In depleted medium, nontransformed cells enter a resting state and stop DNA replication. Those few cells which continue DNA replication after 24 hrs in depleted medium (1-3% of the population) had an interorigin "average" distance of 40 μ. In complete medium, where 50% of the nontransformed cells replicate their DNA, the distance was also 40 μ. In depleted medium, transformed cells do not enter a resting state and at 24 hr of depletion, still ∿ 50% of the cells continued DNA replication. Under these conditions, the "average" distance between origins of DNA replication increased to about 60 μ. In complete medium, the distance between origins of DNA replication in transformed cells dropped to about 30 μ. We have interpreted these results as indicating that in normal cells, initiation is the result of a normal initiation protein which recognizes sites on the DNA, separated by ∿ 40 μ. In transformed cells in depleted medium, no normal initiation protein is synthesized, but TA acts as an initiator by recognizing new DNA sites separated from each other by ∿ 60 μ. In complete medium, DNA synthesis is initiated in transformed cells by both the normal initiator and by the TA, hence the decrease in interorigin distance to 30 μ. (Martin and Oppenheim).

(c) A model for the nucleoprotein core of SV40 virions has been constructed based upon the proposed structure of nuclesomes. (Martin)

The significance of this research to biomedical science is that it may lead to a fundamental understanding of some parameters of normal cell growth and how this growth is preturbed on transformation by SV40.

Work in progress includes an attempt to measure the turnover rate of TA in transformed cells, to obtain large quantities of purified TA and to carry out physical-chemical measurements of TA - DNA binding.

Publications:

Martin, R. G., Persico-DiLauro, M., Edwards, C.A.F. and Oppenheim, A.: The molecular basis of transformation by SV40: The semiautonomous replicon revisited. International Union of Biochemistry Meeting, Varnasi, India. In press.

Anderson, J. L., Martin, R. G., Chang, C. and Mora, P.T.: Tumor-specific transplantation antigen is expressed during SV40 lytic infection with wild-type and tsA mutant viruses. Virology 76: 254-262 (1977).

Anderson, J. L., Martin, R. G., Chang, C., Mora, P.T. and Livingston, D.M.: Nuclear preparations of SV40-transformed cells contain tumor-specific transplantation antigen activity. Virology 76: 420-425 (1977).

Anderson, J. L., Chang, C., Mora, P.T. and Martin, R. G.: Expression and thermal stability of Simian virus 40 tumor-specific transplantation antigen and tumor antigen in wild type- and tsA mutant-transformed cells. J. Virol. 21: 459-467 (1977).

Chang, C., Anderson, J.L., Martin, R.G. and Mora, P.T.: Expression of tumor specific transplantation antigen in cell lines transformed by wild type or tsA mutant SV40 viruses. J. Virol. in press.

Tenen, D.G., Martin, R.G., Anderson, J.L. and Livingston, D.M.: Biological and biochemical studies of cells transformed by Simian virus 40 temperature-sensitive gene A mutants and A mutant revertants. J. Virol. 22: 210-218 (1977).

Martin, R. G., Persico-DiLauro, M., Edwards, C.A.F. and Oppenheim, A.: The molecular basis of transformation by Simian virus 40. Proceedings of the Symposia, Miami, Florida, Pub. Academic Press. In press.

Martin, R. G. and Oppenheim, A.: Initiation points for DNA replication in nontransformed and Simian virus 40 transformed Chinese hamster lung cells. Cell in press.

| SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space) | U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT | PROJECT NUMBER Z01 AM 36101-04 LMB |
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PERIOD COVERED
July 1, 1976 to September 30, 1977

TITLE OF PROJECT (80 characters or less)

Energy conversion in biology, especially muscle contraction

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

| | | | |
|---|---|---|---|
| PI: | Terrell L. Hill | Chief, Section on Theoretical Mol. Biol. | LMB NIAMDD |
| OTHER: | Robert Rubin | Research Physicist | LMB NIAMDD |
| | Yi-der Chen | Visiting Scientist | LMB NIAMDD |

COOPERATING UNITS (if any)

| | |
|---|---|
| Evan Eisenberg | H LB |
| Britton Chance | University of Pennsylvania |

LAB/BRANCH
Laboratory of Molecular Biology

SECTION
Theoretical Molecular Biology

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20014

| TOTAL MANYEARS: | PROFESSIONAL: | OTHER: |
|---|---|---|
| 2-1/5 | 2-1/5 | 0 |

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☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The main accomplishment during the past year has been the completion and publication of a research monograph on the theory of free energy transduction in biology. This gives a unified account of the various well-known examples of free energy transduction such as active transport, muscle contraction, oxidative phosphorylation, etc. The relations between steady-state kinetics and thermodynamics are emphasized.

Work on explicit models of muscle contraction continues. The general approach we are using is set out in a paper with Evan Eisenberg. We hope to conclude the modelling work during the next year.

Extensive mathematical analysis has been carried out on the steady-state kinetics of the respiratory chain enzymes. The latter part of this work, to be published in the near future, was in collaboration with Britton Chance. The same analysis is also applicable to single-file diffusion through membrane channels.

A new project is a study of the effect of enzyme-enzyme interactions on steady-state enzyme kinetics. This generalizes the classical solution and lattice interaction problems of statistical mechanics from equilibrium to steady state. This could be a very extensive area to investigate.

Publications:

Author: Hill, T. L.: Free Energy Transduction in Biology. New York, Academic Press, 1977 (in press).

Simmons, R. M. and Hill, T. L.: Definitions of free energy levels in biochemical reactions. Nature, 263: 615-618, 1976.

Hill, T. L.: Steady-state kinetic formalism applied to multienzyme complexes, oxidative phosphorylation, and interacting enzymes. Proc. Nat. Acad. Sci. USA 73: 4432-4436, 1976.

Eisenberg, E. and Hill, T. L.: A model of muscle contraction. Prog. Biophys. Mol. Biol., in press.

Hill, T. L.: Steady-state kinetics of a linear array of interacting enzymes. In Landman, U. (Ed.): Statistical Mechanics and Statistical Methods in Theory and Application. New York, Plenum Press, 1977 (in press).

Hill, T. L.: Free energy transduction and biochemical cycles. Trends Biochem. Sciences, in press.

Hill, T. L.: Theoretical study of the effect of enzyme-enzyme interactions on steady-state enzyme kinetics. Proc. Nat. Acad. Sci. USA, in press.

Hill, T. L.: Further study of the effect of enzyme-enzyme interactions on steady-state enzyme kinetics. Proc. Nat. Acad. Sci. USA, in press.

| SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space) | U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT** | PROJECT NUMBER ZO1 AM 36102-06 LMB |
|---|---|---|

PERIOD COVERED

July 1, 1976 - September 30, 1977

TITLE OF PROJECT (80 characters or less)

Statistical thermodynamics of polynucleotide - complementary monomer interactions

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: Terrell L. Hill Chief, Sec. on Theoretical Mol. Biol., LMB NIAMDD

COOPERATING UNITS (if any)

None

LAB/BRANCH

Laboratory of Molecular Biology

SECTION

Theoretical Molecular Biology

INSTITUTE AND LOCATION

NIAMDD, NIH, Bethesda, Maryland 20014

| TOTAL MANYEARS: | PROFESSIONAL: | OTHER: |
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☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

This project has been inactive during the current year.



PHS-6040
(Rev. 10-76)

Project No. Z01 AM 36102-06 LMB

Project Description:

The objective here is: (a) to clarify the thermodynamic procedures which should be used in comparing optical, calorimetric, and binding measurements on these systems; and (b) to develop and use, as a prerequisite, a theory of aggregation of monomers in solution.

Extensive work has already been done under both (a) and (b). Applications in the future to particular systems await the completion of joint experimental work by Dr. Philip Ross and Dr. Thomas Schleich (Univ. of Calif., Santa Cruz).

| SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space) | U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT** | PROJECT NUMBER Z01 AM 36103-06 LMB |
|---|---|---|

PERIOD COVERED
July 1, 1976 to September 30, 1977

TITLE OF PROJECT (80 characters or less)
Ion transport across the nerve axon membrane

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: Terrell L. Hill Chief, Sec. on Theoretical Mol. Biol. LMB NIAMDD

OTHER: Yi-der Chen Visiting Scientist LMB NIAMDD

COOPERATING UNITS (if any)
None

LAB/BRANCH
Laboratory of Molecular Biology

SECTION
Theoretical Molecular Biology

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20014

| TOTAL MANYEARS: | PROFESSIONAL: | OTHER: |
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| 4/5 | 4/5 | 0 |

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☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

In our previous work, we were concerned with the question of how to calculate the concentration noise power spectrum of linear kinetic systems when the rate constants of the systems are assumed to be known and the question of how to evaluate the rate constants of the reactions when adequate noise data are available. In our current work, we have extended our analysis to systems with non-linear kinetic reactions. Non-linear chemical reactions are very common in biochemistry. For example, the association-dissociation reactions of multi-subunit systems such as hemoglobins and oligomeric enzymes, or most enzymatic reactions are non-first order reactions. In general, with the use of a "quasi-linearization" approximation, the phenomenological relaxation matrix, the variances, and the resulting noise power spectrum are all expressed in terms of the usual kinetic rate terms of the chemical mass action law. Thus, the calculation of the noise power spectrum becomes very simple as soon as the rate constants of all elementary reactions of the system are known. The formalism can be used as well to evaluate the rate constants of the elementary reactions

of an arbitrary system if adequate noise data are available. Finally, the formalism is very useful in differentiating between an equilibrium and a non-equilibrium steady-state system. Applications to membrane channels such as potassium (or sodium) channels in nerve membranes and ionic channels in lipid bilayers have been discussed.

Publications:

Chen, Yi-der: Fluctuations and noise in non-linear kinetic systems. J. Theoret. Biol. 65: 357-367, 1977.

Chen, Yi-der: The irreversible thermodynamic approach to the fluctuations in chemical reactions. J. Chem. Phys. 66: 2431-2434, 1977.

Chen, Yi-der: Noise analysis in kinetic systems and its application to membrane channels. Adv. Chem. Phys., in press.

Chen, Yi-der: Noise analysis and channel properties. Annals New York Acad. Sci., in press.

Annual Report of the

Mathematical Research Branch

National Institute of Arthritis, Metabolism, and Digestive Diseases

A model of diffusion-reaction kinetics describing the oxygen facilitated diffusion in the presence of hemoglobin or myoglobin has been reported previously. The extensive experimental observations of Wittenberg on the diffusion of oxygen through solutions of hemoglobin or myglobin supported by a millipore membrane are being analyzed. The physicochemical parameters needed to be used in conjunction with the above model are now available through the work of Wittenberg, Hoofd, Goldstick and others. The qualitative as well as quantitative agreement between the experimental and theoretical results obtained so far, seems to indicate that the experimentally observed facilitated diffusion can be interpreted as being effected by the added diffusion of a protein carrier. (Dr. J. Gonzalez-Fernandez and Mrs. S. Atta)

A simplified description of the facilitated diffusion of oxygen that was reported in a previous year has been further developed. A system of two non-linear algebraic expressions incorporating the concentration of bound oxygen at both faces of the slab have been derived. These results are used to estimate the facilitated diffusion as well as to study its dependence on the parameters of the system. (Mrs. S. Atta and Dr. J. Gonzalez-Fernandez).

A nearest neighbor algorithm for a moderate sized collection of points in a high dimensional space has been developed. Previous methods, although efficient for very large files of low dimensionality exhibit a marked deterioration of performance in 8-dimensional spaces, and their behavior has not been explored, either theoretically or experimentally, in higher dimensional spaces. The efficiency of the present method is independent of dimensionality of the space, and in the worst case (equal variance of coordinates in all dimensions) makes possible a saving of 25% of computing time over brute force methods. Computing the distance from the query point to each point in the space and sorting distances is the brute force method. Efficient algorithms substitute some more or less simple rule by which some distance computations maybe eliminated. The present method projects the n dimensional space onto an r dimensional subspace, $r < n$, and uses distances in r space as a part of a cutoff criterion. The optimum subspace is found by a suitable rotation of axes, and the optimum dimensionality r, lies between .4n and .6n. This method is feasible only for files of a size comparable to that of the high speed memory of the computer used. However the rapid advances of high speed memory technology makes this condition less restrictive. (Ms. R.B. Marimont, Associate member, MRB).

Mathematical solutions (steady state and transient) have been obtained (W. Rall) for significant generalizations of a problem concerning the spatio-temporal distribution of membrane potential in response to a current step applied to a lumped neuron soma attached to several dendritic trees. Earlier solutions assumed uniform passive membrane everywhere; the new problem explicitly includes complication by steady synaptic membrane conductance and/or at the dendritic terminals. The solution uses generalized Fourier coefficients

(based upon a modified orthogonality described by Churchill) and a complicated transcendental equation whose roots define changed values for the time constants. Computations with these results can be used for comparison with experiments cited next. Drs. W. Rall and R. Miller in collaboration with Drs. P. G. Nelson and R. MacDonald (both of NICHD, BB) have begun analysis of experimental transients obtained with intracellular microelectrodes in several types of tissue cultured neurons. They will compare the theoretical parameters so obtained with those based upon extensive anatomical information also available for the same particular neurons. Also, Drs. Rall, Rinzel and Miller have begun a collaborative effort with Dr. D. L. Alkon (of NINCDS, LB, Woodshole) to develop mathematical models that would correspond to known anatomy and physiology of the relatively simple visual system of a nudibranch mollusc. (Dr. W. Rall,).

Several aspects of repetitive axonal neuroelectric activity have been formulated and analyzed; these include impulse propagation as well as small amplitude and other maintained oscillations. For the case of a steady current stimulus, I, injected at a single location, we have characterized thresholds, critical values $I_1$, $I_2$ of I at which the adapted response switches from steady state to repetitive activity, and have obtained stimulus-response curves of firing frequency $\omega$ versus I. The threshold problem is treated analytically for a simple, yet nonlinear, FitzHugh-Nagumo nerve conduction (cable) equation by using methods of linear stability and bifurcation theory. For $I=I_1$, $I_2$ the unique spatially decremented steady state loses or gains its stability and small amplitude time periodic solutions emerge. Dependence of $I_1$, $I_2$ on model parameters is thoroughly described and qualitative differences between the point-stimulated case and the space and current-clamped case are exposed and interpreted. For the repetitive firing regime, perturbation methods as above do not apply. The initiation and propagation of action potential trains and their $\omega$-I relation are described by numerical integrations of the excitable cable equations. Results for a cubic FitzHugh-Nagumo equation reveal hysteresis and bifurcation phenomena in the $\omega$-I curve which include high frequency cut-off and jumps in $\omega$, by rational factors, at certain values of I. In addition the results illustrate the threshold properties described above and verify that for I near $I_2$ the small amplitude maintained oscillations are stable. Further simulations for a non-uniform axon demonstrate that geometry can significantly influence impulse conduction during repetitive firing and can lead to total conduction block or intermittent conduction.

As the stimulus-response calculations illustrate, during steady repetitive firing under localized maintained (or periodic) stimulation, the solutions to nerve conduction equations approach periodic traveling waves asymptotically far from the stimulation region. These waves and their dispersion relation, propagation speed versus frequency, can be determined as particular solutions to the cable equations without regard for the stimulus. We have developed, implemented, and tested an accurate and efficient numerical method to determine the periodic wave trains and their dispersion relation. It is being applied to the Hodgkin-Huxley and cubic FitzHugh-Nagumo nerve conduction models to study their dispersive characteristics and dependence of these on model parameters. The method should find wider use for reaction-diffusion models in other contexts.

Discussions with Dr. D. L. Alkon (LB, NINCDS, Woods Hole) on neuroanatomy and sensory neural processing in a nudibranch mollusc have motivated initial stages of modeling the responses and interactions of small groups of neurons. A preliminary two-cell model to study the effect of brief visual stimuli has been formulated and implemented on the computer. (Dr. J. Rinzel).

Studies are being conducted to gain insight into the nature of the electrical behavior of cells whose function includes transduction, integration and transmission of electrical signals. This work consists of mathematical treatment of problems related to details of specific experiments as well as more general theoretical considerations.

Two problems related to specific experiments are currently being investigated. One is the estimation of the electrical parameters that determine the passive electrical response of neuron cell bodies to stimuli originating in the periphery. This is viewed as a necessary first step for understanding the relation between the form of a neuron and its function. The other is the construction of a mathematical model of the visual system of the nudibranch mollusk Hermissenda crassicornis. A mathematical model has been devised for Hermissenda photoreceptors, including kinetics of phototransduction, electrically active response to light and synaptic interaction with neighboring photoreceptors.

More general questions of the nature of propagation of electrical impulses in excitable tissue are also being studied. A numerical method has been devised and implemented for finding the form of periodic traveling wave solutions to the FitzHugh-Nagumo and Hodgkin-Huxley models of nerve cells. This method is currently being used to study dispersion phenomena in these equations. Use is made of simple models and analytical methods to study various aspects of active propagation of electrical impulses in heart and nerve. (Dr. R. Miller)

The following is a summary of the individual and collaborative efforts of Dr. J. Stephenson and Mr. R. Mejia, NLHBI, Associate Members MRB; Drs. B. Kellogg and R. Tewarson, Consultants, NLHBI; Dr. A. Tsan, Guest Worker, NLHBI, Dr. R. Huss, Guest Worker, NIAMDD, Dr. A. Weinstein, Staff Fellow, NLHBI.

During the past year work has continued on the theory of transport and flow process in epithelia and the kidney. Areas of current work include (1) the thermodynamic analysis of flow processes, (2) the qualitative analysis of equations of describing kidney models, (3) the development of analytical solutions of kidney models and (4) the general theoretical analysis of numerical methods.

Specifically: Earlier work on the contribution of viscous dissipation to entropy production in a network of flow tubes has been written up and accepted for publication (Weinstein). In our study of kidney models it has been discovered that for certain choices of parameters, solutions either do not exist or are nonunique (Kellogg & Stephenson). The conditions sufficient to guarantee existence and uniqueness have been established for certain models (Garner & Kellogg). The general analytic theory of multinephron cortical core models has been developed and published (Stephenson). The error analysis of our numerical methods has been greatly improved (Kellogg, Tsan & Tewarson).

The multinephron model has been used to analyze the effect of urea cycling on the concentrating mechanism. It has been confirmed that the inner medulla can concentrate with no active transport out of the AHL. We have also found that the ratio of cortical to juxtamedullary nephrons is a very critical parameter (Mejia & Stephenson).

Water transport in a single solute model of a leaky epithelia (e.g. proximal tubule) that includes both cellular and paracellular pathways has been studied. It has been found that in many respects the steady state performance of the system is not affected by the cellular compartment. This probably reflects the fact that in a single solute model the problem of osmotic regulation of the cellular compartment is in part finessed (Huss).

| SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space) | U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT** | PROJECT NUMBER Z01 AM 13,000-04 MRB |
|---|---|---|

PERIOD COVERED
July 1, 1976 to September 30, 1977

TITLE OF PROJECT (80 characters or less)

Mathematics of kinetics and reaction-transport systems

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: John Z. Hearon Chief, MRB MRB NIAMDD

COOPERATING UNITS (if any)

LAB/BRANCH
Mathematical Research Branch

SECTION

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20014

| TOTAL MANYEARS: | PROFESSIONAL: | OTHER: |
|---|---|---|
| 1.6 | 1.0 | .6 |

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES XX (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

This project continues to focus upon applications of linear algebra, matrix and vector space methods to problems in biology. Applications are heavily involved in compartmental analysis; linear control theory, and generalized kinetics. No small part of the project is devoted to meeting purely mathematical problems which arise on their proper abstract grounds.



PHS-6040
(Rev. 10-76)

Certain qualitative properties of solutions of differential equations, compartmental models in particular, can be assessed by considering some norm of the solution vector. Certain norms other than the Euclidean norm are more useful and in some cases (where the solution vector is inherently nonnegative) more natural. Given a particular norm, we are frequently confronted with certain operator or matrix questions: Which transformations leave the norm unchanged and which matrix norms are consistent with the vector norm. The following represent results which are relevant to those kinds of investigations.

1. Norm-isometries are Diagonable.

The complete characterization of a norm-isometry for an arbitrary vector norm is presently possible only for absolute norms. It has been shown that for an arbitrary norm, each member, K, of the group of isometries has the following properties: (i) For any $\lambda$ in the spectrum, $|\lambda| = 1$. (ii) For any complex scalar $\alpha$, $K-\alpha I$ has disjoint range and null space. It is an immediate consequence of (ii) that K is diagonable. This being so, (i) then implies that K is similar to a unitary matrix. Finally it follows from (i), that the only normal norm isometries are the unitary matrices.

2. Invariance of Ellipsoidal Norms.

Let $\phi$ be an arbitrary ellipsoidal norm. Let $\psi$ be a norm such that $\psi$ is invariant under $\phi$-isometries. Then $\psi=\beta\phi$. Thus if the norm-isometries of a given norm are a subgroup of the norm-isometries for an ellipsoidal norm, the given norm is ellipsoidal. In particular every unitarily invariant norm is (up to a constant multiple) the Euclidean norm.

3. Matrix Norms Invariant under Norm-isometries.

Let N be a multiplicative matrix norm, $\phi$ an ellipsoidal norm and $S$ the group of $\phi$-isometries. If N is invariant under $S$ then N is consistent with $\phi$. In particular the important class of unitarily invariant matrix norms is consistent with the Euclidean norm. If $N(I)=1$ and N is invariant under K, then if N and $\phi$ are consistent we have $K \in S$. Thus a matrix norm invariant under $S$ is consistent only with $\phi$ (or multiples of $\phi$).

Publications:

Hearon, J. Z.: Bounds on the roots of a polynomial. Math. Mag. 49, #5: 240-242, 1976.

Hearon, J. Z.: Nonsingular solutions of TA - BT = C. Linear Algebra and Appl. 16: 57-63. 1977.

Hearon, J. Z.: Minimum polynomials and control in linear systems. J. Res. Nat. Bur. Standards, 1977 (in press).

| SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space) | U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT** | PROJECT NUMBER Z01 AM 13,001-04 MRB |
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PERIOD COVERED
July 1, 1976 to September 30, 1977

TITLE OF PROJECT (80 characters or less)

Mathematical formulations and analysis relevant to experimental neurophysiology.

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

| | | | |
|---|---|---|---|
| PI: | Wilfrid Rall | Senior Research Physicist | MRB NIAMDD |
| OTHER: | John Rinzel | Research Mathematician | MRB NIAMDD |
| | Robert Miller | Staff Fellow | MRB NIAMDD |

COOPERATING UNITS (if any)

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| TOTAL MANYEARS: | PROFESSIONAL: | OTHER: |
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| 2.0 | 1.7 | 0.3 |

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☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

For several years, this project has been concerned with constructing, developing and testing a group of interrelated mathematical models, relevant to experimental neurophysiology and neuroanatomy. Together these models provide a theory that can account for various sequences of events in the soma and dendritic branches of a single neuron, and for field potentials generated by certain cortical populations of neurons. Computational experiments performed with these models provide theoretical predictions that have been compared with experimental results obtained by colleagues with motoneurons of cat spinal cord, and with the mitral cell and granule cell populations of rabbit olfactory bulb. Resulting interpretations contribute to understanding of dendritic synaptic input and of dendro-dendritic synaptic interactions.

These results have appeared in the Biophysical Journal and in the Journal of Neurophysiology; comprehensive chapter, entitled "Core conductor theory and cable properties of neurons" in The Nervous System, Vol. I, Cellular Biology of Neurons, edited by E. R. Kandel, (published by the American Physiological Society 1977 in press) summarizes and explains many of these results.

1. Mathematical solutions (steady state and transient) have been obtained for significant generalizations of an earlier problem (Rall 69). The problem concerns the spatio-temporal distribution of membrane potential in response to a current step applied to a lumped neuron soma attached to dendritic trees represented as an equivalent cylinder. Earlier solutions assumed that the nerve membrane had uniform passive properties everywhere. The new problem explicitly includes complication by steady synaptic membrane conductance and/or steady injury conductance located at the soma and/or at the dendritic terminals. The solution uses generalized Fourier coefficients (based upon a modified orthogonality described by Churchill) and a more complicated transcendental equation whose roots provide modified eigenvalues which define changed values for the time constants. Computations with these results can be used for comparison with experiment.

2. (With Dr. Miller) and (with Drs. P.G. Nelson and R. MacDonald, both of NICHD, BB). Analysis of experimental transients obtained with intracellular microelectrodes in several types of tissue cultured neurons. In spite of difficult data processing problems, we seek best fits of these data to the theoretical functions of item (1). We will compare the theoretical parameters so obtained with those obtained from the extensive anatomical information also available for the same particular neurons.

3. (With Drs. Rinzel and Miller) and (with Dr. D.L. Alkon, of NINCDS, LB, Woodshole). We have begun a collaborative effort to develop mathematical models that would correspond to known anatomy and physiology of the relatively simple visual system of a nudibranch mollusc.

4. Significant theoretical arguments and explanations were prepared and added to the Klee-Rall MS (between acceptance and publication). These provide a more explicit theoretical explanation and validation of the "potential divider model" that had earlier (Rall and Shepherd, 1968) been based partly upon physical intuitive considerations. The complicated three dimensional flow of (secondary) current outside the synchronously activated cortical region can be treated as many tubes of current which flow through resistances which are electrically in parallel; for equipotential outer and inner cortical surfaces, these parallel resistances can be lumped into the simple circuit elements of the potential divider model. Also, explanations were provided to show how the superposition of two or more potential divider calculations can be used to account for certain cases of more complicated neuronal activity.

Publications:

Rall, W.: Core conductor theory and cable properties of neurons. In Kandel, E. R. (Ed.): The Nervous System, Vol. I, Cellular Biology of Neurons. Washington, D.C., Am. Physiol. Soc., 1977 (in press).

Klee, M. and W. Rall: Computed potentials of cortically arranged populations of neurons. J. Neurophysiol. 40:647-666, 1977.

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PERIOD COVERED
July 1, 1976 to September 30, 1977

TITLE OF PROJECT (80 characters or less)

Mathematical description of the substrates transport in the capillary-tissue structures.

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

| | | | |
|---|---|---|---|
| PI: | Jose M. Gonzalez-Fernandez | Research Mathematician | MRB NIAMDD |
| OTHER: | Susie E. Atta | Mathematician | MRB NIAMDD |

COOPERATING UNITS (if any)

LAB/BRANCH
Mathematical Research Branch

SECTION

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20014

| TOTAL MANYEARS: | PROFESSIONAL: | OTHER: |
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CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

To develop mathematical models of the blood flow and transcapillary exchanges in capillary networks. An effort is being made to incorporate in the models the histological structure of capillary networks as well as different flow patterns from available experimental information. In this model the extraction of substrates with different chemical kinetics at the tissue site will be described. It is expected that this could be used in experimental situations where the extraction of different substrates are measured simultaneously, thus helping to infer the flow pattern features of the microcirculation. In particular a model of the diffusion-consumption of oxygen in striated muscle containing myoglobin (facilitated diffusion) is being developed and pertinent numerical results examined.

A model of diffusion-reaction kinetics describing the oxygen facilitated diffusion in the presence of hemoglobin or myoglobin has been reported previously. The extensive experimental observations of Wittenberg on the diffusion of oxygen through solutions of hemoglobin or myoglobin supported by a millipore membrane are being analyzed. The physicochemical parameters needed to be used in conjunction with the above model are now available through the work of Wittenberg, Hoofd, Goldstick and others.

The qualitative as well as quantitative agreement between the experimental and theoretical results obtained so far, seems to indicate that the experimentally observed facilitated diffusion can be interpreted as being effected by the added diffusion of a protein carrier.

A simplified description of the facilitated diffusion of oxygen that was reported in a previous year has been further developed. A system of two nonlinear algebraic expressions incorporating the concentration of bound oxygen at both phases of the slab have been derived. These results are used to estimate the facilitated diffusion as well as to study its dependence on the parameters of the system.

Publications: None.

| SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space) | U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT** | PROJECT NUMBER Z01 AM 13,004-03 MRB |
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PERIOD COVERED
July 1, 1976 to September 30, 1977

TITLE OF PROJECT (80 characters or less)

Mathematical description of cellular neuroelectric signal transmission.

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

| | | | |
|---|---|---|---|
| PI: | John Rinzel | Research Mathematician | MRB NIAMDD |
| OTHER: | Wilfrid Rall | Senior Research Physicist | MRB NIAMDD |
| | Richard FitzHugh | Research Physicist | LB NINCDS |
| | Robert Miller | Staff Fellow | MRB NIAMDD |

COOPERATING UNITS (if any)

Laboratory of Biophysics, NINCDS

LAB/BRANCH
Mathematical Research Branch

SECTION

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20014

| TOTAL MANYEARS: | PROFESSIONAL: | OTHER: |
|---|---|---|
| 1.9 | 1.6 | .3 |

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

This project continues to focus on the formulation, analysis, and biophysical interpretation of mathematical models which describe various aspects of neuroelectric signaling for individual neurons. Among the topics of current interest are:

(i) integration of synaptic input delivered to the soma and dendritic branches of a neuron;

(ii) steady propagation of action potentials along axons;

(iii) stimulus-response and threshold properties for repetitive firing of action potentials;

(iv) excitable membrane characteristics and firing patterns of bursting pacemaker neurons.

Mathematical models of these phenomena involve systems of linear and nonlinear ordinary differential equations and parabolic partial differential equations. Solutions and their mathematical stability are determined by analytical and numerical methods. One aspect in the approach of this project is to expose the qualitative mathematical structure for classes of models by exploiting simple, yet reasonable, equations.

1. It is typically found that the adapted electrical response of a neuron may switch from steady state to repetitive activity (or vice versa) as stimulus amplitude is slowly varied. This threshold phenomenon has been analytically revealed for a simplified FitzHugh-Nagumo (FHN) nerve conduction model in which the axon is stimulated at a single location by a steady current I. Mathematical methods of linear stability and bifurcation theory are used to characterize critical values $I_1$, $I_2$ of I at which the unique spatially decremented steady state loses or gains its stability. For $I=I_1$, $I_2$, small amplitude time periodic solutions bifurcate from the steady state. For the simplified model we explicitly describe the dependence of $I_1$, $I_2$ on model parameters such as a temperature like rate coefficient. Qualitative differences between the point-stimulated case, studied here, and the corresponding space-clamped problem are exposed. For example, results suggest that for a certain temperature range a point-stimulated axon may adapt to a slowly rising current but a space-clamped axon will not. Our results further support experimental observations that frequency of repetitive activity jumps discontinuously from zero at critical I values and that as I increases, the site of impulse generation shifts farther away from the stimulus location. This work was described in a contributed talk at the 1977 Biophysical Society Meeting and a manuscript has been submitted to the Journal of Mathematical Biology. For the simplified model, which has a piecewise linear current-voltage law for the excitable membrane, we have not determined the stability of the bifurcating periodic solutions. Results for other FHN membrane models are now being obtained (in collaboration with Dr. J. Keener, Math. Dept., U. of Arizona); these include stability calculations for the bifurcating periodic solutions.

2. (With R. Miller) Mathematical results for a simple nerve conduction equation suggest that during repetitive active the propagation speed $\theta$ of a periodic train of action potentials depends on the firing frequency $\omega$. To determine the dispersion relation $\theta=\theta(\omega)$ for other nerve conduction equations we have developed an accurate and efficient numerical method. The technique has been tested successfully against explicit analytical results for the simple nerve model. As a special case, our method can also be applied to determine the repetitive firing pattern for a space and current-clamped model. Since such periodic solutions are easy to obtain by a different numerical method, we have compared results for the Hodgkin-Huxley equation from the two approaches as a further test of our method. We are now generating dispersion curves for the Hodgkin-Huxley and also the FHN models and intend to study their dependence on various physiological parameters.

3. Because the methods in paragraph 1 are based on perturbations about the steady state they do not yield descriptions for the generation and propagation of action potentials away from the stimulus location. For this, I numerically integrate the full nonlinear cable equations with a step of current applied at a single location. Such calculations, for the FHN model with a cubic current-voltage law, first illustrate the bifurcation and threshold properties outlined above (with stable small amplitude oscillations about the steady state for I near $I_2$) and additionally yield the firing frequency versus current strength, $\omega$-I, relation for the axon model. In the repetitive firing regime, the propagation speed of a periodic pulse train is found to satisfy the dispersion relation as determined in paragraph 2. The simulations reveal that hysteresis phenomena can occur in the $\omega$-I relation and also show that the

frequency of the transmitted train may discontinuously drop by rational given location, demonstrate the influence of geometry on impulse conduction during repetitive firing. One may observe, depending upon frequency of the approaching action potential train and magnitude of diameter jump, successful conduction, total conduction failure, or intermittent conduction (frequency demultiplication) through the nonuniform region. These results, along with descriptions of numerical methods suitable for nerve conduction and general nonlinear reaction-diffusion equations, were presented by invitation at the Houston Conference on Nonlinear Diffusion, 1977 (paper in press).

4. (with R. FitzHugh, LB, NINCDS) Additional numerical results for our simple model of neuronal bursting activity reveal sensitive dependence of burst firing patterns on parameter values. Although the model is deterministic, some patterns are quite irregular with long periods and appear nearly random. While similar features ("chaos") have been found for other mathematical dynamical systems, they do not appear to be characteristic of the stable patterns observed in pacemakers like Aphysia R15. Our firing patterns however, do share some features with "skip-run" patterns seen in partially decalcified squid axon experiments.

5. (with W. Rall, R. Miller) and (Dr. D. L. Alkon, LB, NINCDS, Woods Hole) Discussions with Dr. Alkon of experimental results on sensory neural processing in a nudibranch mollusc have motivated initial stages of modeling the responses and interactions of small groups of neurons. A preliminary two-cell model to study the effect of brief visual stimuli has been formulated and implemented on the computer. Each model neuron includes transduction of sensory input to generator potential, a trigger zone for action potential initiation, and branching processes where mutual synaptic inhibitory interactions occur. Simulations and ongoing discussions with Dr. Alkon continue to sharpen the focus of theoretical questions and to suggest additional experiments to deduce biophysical mechanisms and parameter values for the model.

Publications:

1. Rinzel, J.: Nerve signaling and spatial stability of wave trains, in Structural Stability, the Theory of Catastrophes, and Applications in the Sciences (P. J. Hilton, ed.) Lecture Notes in Mathematics 525, Springer-Verlag, New York, 1976.

2. Rinzel, J.: Integration and propagation of neuroelectric signals, in MAA Study in Mathematical Biology (S. A. Levin, ed.), Math. Assoc. America, Washington, D.C. (in press).

3. Rinzel, J.: Repetitive nerve impulse propagation: numerical results and methods, in Topics in Nonlinear Diffusion (W. E. Fitzgibbon, H. F. Walker, eds.) Pitman, (in press).

| SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space) | U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT** | PROJECT NUMBER Z01 AM 13,011-02 MRB |
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PERIOD COVERED
July 1, 1976 to September 30, 1977

TITLE OF PROJECT (80 characters or less)
Mathematical models of electrical impulse propagation in excitable tissue

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

| | | | |
|---|---|---|---|
| PI: | Robert Miller | Staff Fellow | MRB NIAMDD |
| OTHER: | Wilfrid Rall | Senior Research Physicist | MRB NIAMDD |
| | John Rinzel | Research Mathematician | MRB NIAMDD |

COOPERATING UNITS (if any)

LAB/BRANCH
Mathematical Research Branch

SECTION

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20014

| TOTAL MANYEARS: | PROFESSIONAL: | OTHER: |
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CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

We wish to use Mathematical Models to gain insight into the nature of transduction, integration and propagation of electrical signals in nerve. To this purpose we study both the theoretical consequences of general mathematical models of nerve cells, such as the FitzHugh-Nagumo equations, and the data gathered in certain specific experiments. We have used both computational and analytical methods to investigate the behavior of several well known mathematical models of impulse transmission in nerve cells. We have also developed models and computational procedures for analyzing the results of electrophysiological experiments.



PHS-6040
(Rev. 10-76)

1. (with Dr. Rinzel) The FitzHugh-Nagumo and Hodgkin-Huxley models of active electrical behavior of nerve cells are known to exhibit periodic traveling wave solutions. These solutions correspond to periodic trains of pulses propagating along the cell. We have developed, tested and implemented a numerical method to find such solutions. We are now using this method to investigate the nature of these solutions, with particular regard to the dispersion relation, i.e. the relation of the speed of the wavetrain to its frequency, and the dependence of this relation upon the parameters in the model.

2. Interesting nonlinear wave propagation phenomena have been observed experimentally in cardiac Purkinje fibers, which form the specialized electrical conduction system of the heart. These effects, such as conduction of one pulse through a damaged region for every two or three incident pulses or conduction through a damaged region in only one direction, are thought by the experimenters to be intimately connected with the nature of certain heart disorders. In my doctoral dissertation I proposed a mathematical model of this system and was able to reproduce some of the experimentally observed effects by numerical simulation. Through this model I hope to provide a conceptual framework for understanding this system. A manuscript has been prepared and is essentially finished, and earlier results are being extended.

3. (with Dr. Rall and with Drs. Nelson and MacDonald, both of Behavioral Biology Br., NICHD) Computational procedures for analyzing experimental transients from intracellular recordings of tissue cultured neurons have been developed and are being tested. We wish to fit the experimental data with the theoretically predicted responses, and correlate the parameters so estimated with morphological data from these neurons. Ultimately we see this work as a necessary first step toward a systematic understanding of the relation between the geometry of a neuron and its functional characteristics.

4. (with Drs. Rall and Rinzel, and with Dr. D. L. Alkon of LB, NINCDS, Woods Hole, Mass.) Mathematical models of the visual system of the nudibranch mollusk Hermissenda crassicornis are being developed. A model of the response of the photoreceptor to flashes of light has been implemented computationally. Work is now in progress toward a model the interactions between the photoreceptors in the eye, and our estimates of the parameters in the model of a single photoreceptor are being improved.

Publications: None.

Annual Report of the Arthritis and Rheumatism Branch
National Institute of Arthritis, Metabolism and Digestive Diseases

## I. Clinical Studies

Dr. John H. Klippel, newly appointed Senior Investigator in the Branch, has begun the development of a comprehensive clinical database which lends itself to computerized storage and retrieval of information. It is being applied initially to the ongoing Branch studies of systemic lupus erythematosus (SLE) and does allow for the regular entry of new information on individual cases.

Studies of the management of the nephritis of systemic lupus continue. Patients with the diffuse renal disease are invited to enter a plan calling for random assignment to prednisone only, three monthly intravenous cyclosphosphamide and continuing prednisone or small oral doses of cyclophosphamide and azathioprine with continuing prednisone. Patients in all groups are doing well. Follow-up studies of earlier patients using the same drugs in different schedules and forms continue. Currently most of these patients, long known to us, are receiving a second renal biopsy in order to permit more precise definition of their current status.

In the course of such work many other observations are made, some raising questions which can be submitted to study. The now well-documented effect of high doses of aspirin reducing the creatinine clearance was examined in detail by Drs. Kimberly and Plotz. The effect is extremely important if one wishes to study, for example, the long-term effects of cyclophosphamide on creatinine clearance. The aspirin induced changes appear to be of a temporary nature without permanent damage to renal function.

Dr. Steinberg's studies on the mechanisms of disease have shown a correlation of anti-lymphocyte antibodies, primarily anti-T cell, with clinical measures of disease activity. He and his associates carried out a study of swine influenza vaccination in 40 patients with SLE; the vaccine did not cause an exacerbation of the underlying disease.

Dr. van Boxel has found so-called Fc receptors on T-lymphocytes. There is some reason to believe that the class of Fc recognized, that is, IgG or IgM, is related to the functional character of the cell, that is, helper, suppressor or other.

Dr. Plotz and Dr. Frank (NIAID) have been observing the clearance of specifically sensitized autologous red cells in patients with SLE. To date there appears to be some defect in the patients which results in slowed and less complete clearance. The studies may be able to explain the deposition of extraordinary amounts of immune complexes in vessel walls in SLE. It is these depositions which are believed to incite the nephritis.

Antibodies to nucleic acids are critical to these same complexes and have been of long standing interest to Dr. Steinberg. He and his associates have found increased prevalence of these antibodies in patients with myasthenia gravis, in patients with IgA deficiency and in certain American Indian groups

where the presence of such antibodies is well correlated with an extraordinary incidence of SLE. Dr. Akizuki has been responsible for the development of a radioimmunoassay of antibodies to a soluble nuclear antigen. These antibodies are present in Sjogren's syndrome as an entity but, surprisingly, are not found when the Sjogren's syndrome is accompanied by rheumatoid arthritis.

Dr. van Boxel and Dr. Torretti have carried through a difficult series of experiments which strongly suggest that the neutropenia of Felty's syndrome is due to an anti-neutrophil antibody. With complement the material is cytotoxic from neutrophils at 37°. It is of IgG class and can be demonstrated on the surface of the neutrophil by immunofluorescence.

II. Animal Studies

The NZB/W hybrid mouse lupus is a focus of substantial interest and work and has produced insights apparently directly applicable to human disease. As the mice age nephritis begins to express itself, earlier in the females. It has been found that, as the animals age, T suppressor cell function is lost and this is thought to permit the overproduction of antibodies which is so characteristic of the full-blown syndrome. No such loss of T-suppressor activity is observed in control mouse strains. Castration of male NZB/W hybrids causes acceleration of their disease, a pace which can be slowed by the administration of testosterone.

Drs. Steinberg, Klassen, Williams and Reinertsen and Dr. Gerber (Rehab, CC) have demonstrated chlorambucil to be ineffective in controlling the murine disease but have also found very substantial benefit from an anti-viral agent, ribavirin. The latter appears to have little if any immunosuppressive potency but the reduction in glomerular immune complexes in the mice is comparable to that which can be obtained with cyclophosphamide. The group was also able to confirm earlier findings of a less than 10,000 molecular weight suppressor substance in the supernatant of Concanavalin A stimulated lymphocytes; the material, injected repeatedly into NZB/W mice, prolonged survival.

III. Fundamental Studies

Dr. Neufeld's group has found that the receptor-mediated pinocytosis of iduronidase can be inhibited by mannose 6-phosphate, a finding which may lead to definition of the missing or masked portion of the enzyme in the mucolipidoses. A microassay for iduronate sulfatase has been applied to individual hair follicles, each follicle often representing a single clone of epithelial cells. The hair follicles of Hunter syndrome heterozygotes are of two types, one with and the other without the enzyme activity. If confirmation can be obtained, the system may become the long-sought scheme for detecting such heterozygotes.

Intensive study of IgE receptors of rat basophilic leukemia and of mouse mastocytoma cells have continued. The receptor has been shown to be univalent, to have mobility on the cell surface, and to have a molecular weight of about 130,000. Antisera to the receptor have been prepared and should be

able to aggregate the receptor and trigger the cell. If this proves to be possible, IgE would be relegated to a relatively passive role, serving as a bridge between the antigen (aggregator) and the receptor on the cell surface.

Dr. Plotz has continued his work on model immune complexes and has succeeded in preparing them with monomeric, dimeric, or trimeric forms of antibody. The larger the complex, the more rapidly they disappeared from the circulation of the experimental animal.

| SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space) | U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT** | PROJECT NUMBER Z01 AM 41000-08 ARB |
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PERIOD COVERED

July 1, 1976 to September 30, 1977

TITLE OF PROJECT (80 characters or less)

Controlled study of cyclophosphamide and azathioprine in systemic lupus erythematosus with nephritis

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

| | | | |
|---|---|---|---|
| PI: | J.L. Decker | Chief, Arthritis and Rheumatism Branch | ARB NIAMDD |
| | A.D. Steinberg | Senior Investigator | ARB NIAMDD |
| OTHER: | P.H. Plotz | Senior Investigator | ARB NIAMDD |

COOPERATING UNITS (if any)

None

LAB/BRANCH

Arthritis and Rheumatism Branch

SECTION

Connective Tissue Disease

INSTITUTE AND LOCATION

NIAMDD, NIH, Bethesda, Maryland 20014

| TOTAL MANYEARS: | PROFESSIONAL: | OTHER: |
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| ¼ | ¼ | 0 |

CHECK APPROPRIATE BOX(ES)

☒ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Patients with diffuse glomerulonephritis and systemic lupus erythematosus satisfying criteria for active renal disease are offered a therapeutic study. The patients are randomized to different treatment programs which include the following drugs: corticosteroids, azathioprine and cyclophosphamide.

Follow-up visits occur every six months and the patients are followed until death or dialysis. Clinical and immunologic assessment are performed. The risks and benefits of each treatment will be evaluated and compared.



PHS-6040
(Rev. 10-76)

Project Description:

Objectives:

Two previous ten week studies by our branch suggested that cyclophosphamide was effective in the treatment of lupus nephritis and that azathioprine was less effective. A long-term study of these patients is being carried out to determine whether or not one therapeutic program is superior over the course of several years. In addition, newer drug programs have been suggested by study in New Zealand mice.

Methods Employed:

Patients meeting predefined criteria for diagnosis of SLE and activity of nephritis have been admitted to the trial. In a baseline control period, the patients were kept on the anti-inflammatory regimen (usually prednisone and aspirin) needed to control extra-renal manifestations. These have been continued into the study period with as little change as possible and with the addition of randomly assigned 1) cyclophosphamide plus azathioprine, 2) intravenous cyclophosphamide or 3) steroids only.

A large number of serological, chemical and urine values are serially obtained and the patients will be followed for life. Immunoglobulin levels, anti-DNA antibodies, and serum complement are among those measures. We will determine time to death or renal dialysis in each treatment program and assess the toxicity as well as efficacy of each program.

Major Findings:

Patients in all groups have done well. There has been no significant differences among the programs; however, longer follow-up will be necessary before any conclusions can be drawn.

Significance to Bio-Medical Research and the Program of the Institute:

If difference in the response of the disease and some of the immunological processes can be detected between different drug programs, it may be possible to draw conclusions with reference to the cell populations involved in systemic lupus erythematosus and thus increase our understanding of the disease process itself. It is expected that the results will permit us to select among treatment programs in clinical management.

Proposed Course:

A long-term follow-up of these patients will be continued, primarily by one week admissions every 6 months.

Publications:

1. Decker, J.L. and Steinberg, A.D.: The current status of cytotoxic drug therapy in rheumatoid arthritis and systemic lupus erythematosus. (In Russian) Terapeuticheskii Arkhiv, 1976 (by invitation) pp 79-92.

2. Gerber, N.L. and Steinberg, A.D.: Clinical use of immunosuppressive drugs. Part II. Drugs 11:90-112, 1976.

3. Steinberg, A.D. and Williams, G.W.: Pharmacological immunosuppression in Clinical Immunology, M. Sampter (ed.), 1977 (In publication).

| SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space) | U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT** | PROJECT NUMBER Z01 AM 41001-06 ARB |
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PERIOD COVERED

July 1, 1976 to September 30, 1977

TITLE OF PROJECT (80 characters or less)

Therapy of SLE: A controlled trial comparing high dose intravenous cyclophosphamide with placebo

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

| | | | |
|---|---|---|---|
| PI: | P.H. Plotz | Senior Investigator | ARB NIAMDD |
| OTHER: | J.L. Decker | Chief, Arthritis and Rheumatism Br. | ARB NIAMDD |
| | A.D. Steinberg | Senior Investigator | ARB NIAMDD |

COOPERATING UNITS (if any)

None

LAB/BRANCH

Arthritis and Rheumatism Branch

SECTION

Connective Tissue Disease

INSTITUTE AND LOCATION

NIAMDD, NIH, Bethesda, Maryland 20014

| TOTAL MANYEARS: | PROFESSIONAL: | OTHER: |
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☒ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

At present, no satisfactory therapy exists for severe manifestations of SLE except for renal disease. Even renal disease does not respond well to conventional therapy when the creatinine clearance is markedly reduced. In this study we will test the efficacy of high dose intravenous therapy with cyclophosphamide for the control of severe SLE unresponsive to a trial of high doses of prednisone (up to 2 mg per kg for 3 weeks).

Project Description:

Objectives:

At present no satisfactory therapy exists for severe manifestations of SLE except for renal disease. Even renal disease does not respond well to conventional therapy when the creatinine clearance is markedly reduced. In this study we will test the efficacy of high dose intravenous therapy with cyclophosphamide for the control of severe SLE unresponsive to a trial of high dose of prednisone (up to 2 mg per kg for 3 weeks).

Methods Employed:

Patients with 1) severe renal disease or rapid deterioration of renal function, 2) sensitivity to azathioprine precluding the CAP protocol, 3) severe extra-renal lupus (seizures, hemorrhage, psychosis, hemolytic anemia, myocarditis, or pericarditis uncontrolled by high-dose prednisone) will be randomly assigned to receive intravenous cyclophosphamide or placebo for 3 initial injections over 6 weeks and then at 3 month intervals. Renal and non-renal patients will be separately randomized. The study will be double-blind: neither the physicians nor patients will know which therapy is being administered. The end point of the study will be death or the need for renal dialysis except that patients well for 1 year may have therapy stopped. The potential hazards and benefits will be explained in full to all patients or their families.

Major Findings:

No patients were entered during the past year.

Significance to Bio-Medical Research and the Program of the Institute:

The therapy of SLE has been under study here for several years. The present study is an attempt to extend the therapy for patients unresponsive to conventional therapy.

Proposed Course:

We plan to enter 20 to 30 patients into the study over the next 3-5 years.

Publications:

None

| SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space) | U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT** | PROJECT NUMBER Z01 AM 41002-12-ARB |
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PERIOD COVERED
July 1, 1976 to September 30, 1977

TITLE OF PROJECT (80 characters or less)

Retrospective Evaluation of Knee Synovectomy in Rheumatoid Arthritis

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: J.L. Decker Chief, Arthritis and Rheumatism Branch ARB NIAMDD

COOPERATING UNITS (if any)

None

LAB/BRANCH
Arthritis and Rheumatism Branch

SECTION
Connective Tissue Disease

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20014

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☒ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

This project has been discontinued in that no new patients will be admitted to it. The admission criteria were so strict that suitable cases were rarely found and, accordingly, the pace of the project was unduly slow.

The twelve patients originally admitted are still in follow-up. The procedure did not alter the course of their disease, either systemically or locally.

The project has been terminated.



PHS-6040
(Rev. 10-76)

| SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space) | U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT** | PROJECT NUMBER Z01 AM 41003-04 ARB |
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PERIOD COVERED

July 1, 1976 to September 30, 1977

TITLE OF PROJECT (80 characters or less)

Synovial changes in rheumatoid arthritis under cyclophosphamide therapy

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: John L. Decker, M.D. Chief, Arthritis and Rheumatism Br. ARB NIAMDD

COOPERATING UNITS (if any)

None

LAB/BRANCH

Arthritis and Rheumatism Branch

SECTION

Connective Tissue Disease

INSTITUTE AND LOCATION

NIAMDD, NIH, Bethesda, Maryland 20014

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

This project has been discontinued. Patients with rheumatoid arthritis are now increasingly rarely given cyclophosphamide therapy because of the multiple side effects produced by the drug. No further patients will be admitted to this study.

This project has been terminated.



PHS-6040

| SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space) | U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT** | PROJECT NUMBER Z01 AM 41004-12 ARB |
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PERIOD COVERED

July 1, 1976 to September 30, 1977

TITLE OF PROJECT (80 characters or less)

Role of infection in rheumatoid arthritis

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: John L. Decker Chief, Arthritis and Rheumatism Br. ARB NIAMDD

COOPERATING UNITS (if any)

None

LAB/BRANCH

Arthritis and Rheumatism Branch

SECTION

Connective Tissue Disease

INSTITUTE AND LOCATION

NIAMDD, NIH, Bethesda, Maryland

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

This project has been completed.



PHS-6040
(Rev. 10-76)

This project has been terminated with one additional publication.

1. Decker, J.L. and Barden, J.A.: Mycoplasma arthritis in the pig. In International Symposium on Infection and Immunology in the Rheumatic Diseases, by D. Dumonde (ed.), Blackwell Scientific Publications, Oxford, 1976, pp 191-193.

| SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space) | U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT** | PROJECT NUMBER Z01 AM 41005-05 ARB |
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PERIOD COVERED

July 1, 1976 to September 30, 1977

TITLE OF PROJECT (80 characters or less)

Cellular and humoral immunity in systemic lupus erythematosus (SLE) undergoing randomized cytotoxic therapy

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

| | | | |
|---|---|---|---|
| PI: | J.L. Decker | Chief, Arthritis and Rheumatism Br | ARB NIAMDD |
| OTHER: | A.D. Steinberg | Senior Investigator | ARB NIAMDD |

COOPERATING UNITS (if any)

None

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SECTION

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| TOTAL MANYEARS: | PROFESSIONAL: | OTHER: |
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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

This project has been terminated.



PHS-6040

| SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space) | U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT** | PROJECT NUMBER Z01 AM 41006-04 ARB |
|---|---|---|

PERIOD COVERED

July 1, 1976 to September 30, 1977

TITLE OF PROJECT (80 characters or less)

Characterization of Infiltrating Lymphocytes in Rheumatoid Synovial Membranes

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

| | | | |
|---|---|---|---|
| PI: | J.A. van Boxel | Visiting Scientist | ARB NIAMDD |
| OTHER: | D. Torretti | Clinical Associate | ARB NIAMDD |
| | J.L. Decker | Chief, Arthritis and Rheumatism Br. | ARB NIAMDD |

COOPERATING UNITS (if any)

None

LAB/BRANCH

Arthritis and Rheumatism Branch

SECTION

Connective Tissue Disease

INSTITUTE AND LOCATION

NIAMDD, NIH, Bethesda, Maryland 20014

| TOTAL MANYEARS: | PROFESSIONAL: | OTHER: |
|---|---|---|
| 0 | 0 | 0 |

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

This project has been terminated.



PHS-6040
(Rev. 10-76)

| SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space) | U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT** | PROJECT NUMBER Z01 AM 41007-03 ARB |
|---|---|---|

PERIOD COVERED

July 1, 1976 to September 30, 1977

TITLE OF PROJECT (80 characters or less)

Cell Surface Immunoglobulins of Lymphocytes in Sjogren's Syndrome

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

| | | | |
|---|---|---|---|
| PI: | John A. van Boxel | Visiting Scientist | ARB NIAMDD |
| OTHER: | Stephen A. Paget | Clinical Associate | ARB NIAMDD |
| | Thomas S. Chused | Senior Investigator | LMI NIDR |

COOPERATING UNITS (if any)

Laboratory of Microbiology and Immunology, NIDR

LAB/BRANCH

Arthritis and Rheumatism Branch

SECTION

Connective Tissue Disease

INSTITUTE AND LOCATION

NIAMDD, NIH, Bethesda, Maryland 20014

| TOTAL MANYEARS: | PROFESSIONAL: | OTHER: |
|---|---|---|
| ½ | ¼ | ¼ |

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

This study has been completed.



PHS-6040
(Rev. 10-76)

| SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space) | U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT | PROJECT NUMBER Z01 AM 41008-03 ARB |
|---|---|---|

PERIOD COVERED

July 1, 1976 to September 30, 1977

TITLE OF PROJECT (80 characters or less)

Characterization of Infiltrating Lymphocytes in Experimental Autoimmune Thyroiditis of the Guinea Pig

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

| | | | |
|---|---|---|---|
| PI: | J.A. van Boxel | Visiting Scientist | ARB NIAMDD |
| OTHER: | S.A. Paget | Clinical Associate | ARB NIAMDD |

COOPERATING UNITS (if any)

None

LAB/BRANCH

Arthritis and Rheumatism Branch

SECTION

Connective Tissue Disease

INSTITUTE AND LOCATION

NIAMDD, NIH, Bethesda, Maryland 20014

| TOTAL MANYEARS: | PROFESSIONAL: | OTHER: |
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| ½ | ¼ | ¼ |

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

This project has been completed.



PHS-6040
(Rev. 10-76)

This project has been completed with the following publication:

1. Paget, S.A., McMaster, P.R.B. and van Boxel, J.A.: Experimental Autoimmune Thyroiditis in the Guinea Pig: Characterization of Infiltrating Lymphocyte Populations. J. Immunol. 117:2267-2269, 1976.

| SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space) | U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT | PROJECT NUMBER Z01 AM 41009-03 ARB |
|---|---|---|

PERIOD COVERED
July 1, 1976 to September 30, 1977

TITLE OF PROJECT (80 characters or less)

Studies of IgD on Cord Blood Lymphocytes and Mouse Lymphocytes

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

| | | | |
|---|---|---|---|
| PI: | J.A. van Boxel | Visiting Scientist | ARB NIAMDD |
| OTHER: | F.D. Finkelman | Research Associate | LI NIAID |
| | R. Asofsky | Chief, Lab. Microbial Immunity | LMI NIAID |
| | W.E. Paul | Chief, Lab. Immunology | LI NIAID |

COOPERATING UNITS (if any)

Laboratory of Immunology and the Laboratory of Microbial Immunity, NIAID

LAB/BRANCH
Arthritis and Rheumatism Branch

SECTION
Connective Tissue Disease

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20014

| TOTAL MANYEARS: | PROFESSIONAL: | OTHER: |
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| ¼ | ¼ | 0 |

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The project has been completed.



PHS-6040
(Rev. 10-76)

This project has been completed with the following publication.

1. Finkelman, F.D., van Boxel, J.A., Asofsky, R. and Paul, W.E.: Cell membrane IgD: Demonstration of IgD on human lymphocytes by enzyme-catalzed iodination and comparison with cell surface Ig of mouse, guinea pig, and rabbit. J. Immunol. 116:1173-1181, 1976.

| SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space) | U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT** | PROJECT NUMBER Z01 AM 41010-03 ARB |
|---|---|---|

PERIOD COVERED
July 1, 1976 to September 30, 1977

TITLE OF PROJECT (80 characters or less)
Effect of purified antibody to IgD on lymphocyte transformation and immunoglobulin production.

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

| | | | |
|---|---|---|---|
| PI: | J.A. van Boxel | Visiting Scientist | ARB NIAMDD |
| OTHER: | S. Broder | Clinical Associate | MET NCI |
| | T.A. Waldman | Chief, Metabolism Branch | MET NCI |

COOPERATING UNITS (if any)
Metabolism Branch, National Cancer Institute

LAB/BRANCH
Arthritis and Rheumatism Branch

SECTION
Connective Tissue Disease

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20014

| TOTAL MANYEARS: | PROFESSIONAL: | OTHER: |
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| ½ | ½ | 0 |

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The effect of purified antibody to IgD was tested for effects on in vitro immunoglobulin synthesis by human blood lymphocytes. Inhibition of Ig production was found at low concentrations of anti-IgD. The Fc fragment of the antibody did not have a role in this suppression. Clear differential effects on Ig production were found between anti-D and anti-M when mitogen stimulated cultures were used.



PHS-6040
(Rev. 10-76)

Project Description:

Objectives:

We were previously the first to demonstrate that IgD, an immunoglobulin class of as yet unknown function, is bound to the surface membrane of an unexpectedly high frequency of human lymphocytes. So as to investigate the biological role of IgD the effects on purified antibody to IgD was tested in various in vitro systems for possible stimulator or inhibitor effects, and compared with the effects of anti-IgM.

Methods Employed:

IgD myeloma protein was purified using DEAE cellulose chromatography followed by G 200 gel filtration. Goat antibody to IgD was purified by affinity chromatograhpy. Anti-IgD was added to cultures of human lymphocytes and to the mixed lymphocyte reaction and effects monitored by standard methods of isotope incorporation. Ig secretion was measured by radioimmunoassay.

Major Findings:

A striking inhibition of the in vitro synthesis of the three major serum heavy chain classes was found to occur when anti-IgD was added to cultures of human peripheral blood lymphocytes. This inhibition was seen at very low levels of added antibody. Inhibitory effects were found to be proportional to the amount of anti-IgD added. $F(ab^1)_2$ fragments of anti-IgD were equally suppressive indicating that the effect was not mediated via the Fc portion of the Ig molecule. Whereas anti-M was suppressive in mitogen stimulated cultures as well, anti-D was not, indicating a differential effect of these two antibodies.

Significance to Bio-Medical Research and the Program of the Institute:

IgD is currently the focus of a great deal of research interest. It is probably the major cell surface immunoglobulin in mammals. However, concepts as to its role in the immune system are as yet entirely speculative. Although the significance of anti-IgD inhibition of Ig synthesis in vitro are not clear at this stage, it may have implications with regard to a receptor function of cell surface IgD. Recent evidence suggests that IgD may play a fundamental role in immunological tolerance.

Proposed Course:

Investigations will be directed at elucidating the significance of the in vitro inhibitor effect of anti-IgD.

Publications:

1. van Boxel, J.A., Broder, S. and Waldman, T.A.: Inhibition of immunoglobulin synthesis *in vitro* by purified antibody to IgD. In Eisvoogel, E. (ed.): Proceedings of the Tenth Annual Leukocyte Culture Conference. Amsterdam, North Holland Publishing Corporation, 1976.

2. Manuscript in preparation.

| SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space) | U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT** | PROJECT NUMBER Z01 AM 41011-04 ARB |
|---|---|---|

PERIOD COVERED
July 1, 1976 to September 30, 1977

TITLE OF PROJECT (80 characters or less)

Binding of Aggregated γ-globulin to T-lymphocytes

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

| | | | |
|---|---|---|---|
| PI: | John A. van Boxel | Visiting Scientist | ARB NIAMDD |
| OTHER: | David L. Rosenstreich | Senior Surgeon | LMI NIDR |

COOPERATING UNITS (if any)

Laboratory of Microbiology and Immunology, NIDR

LAB/BRANCH
Arthritis and Rheumatism Branch

SECTION
Connective Tissue Disease

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20014

| TOTAL MANYEARS: | PROFESSIONAL: | OTHER: |
|---|---|---|
| ½ | ¼ | ¼ |

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Efforts are being directed towards establishing that Fc receptors appear on activated T cells in man. Lymphocytes will be activated in vitro by the mixed lymphocyte reactions, and also by mitogens, and Fc receptors on these activated populations will be compared.



PHS-6040
(Rev. 10-76)

Project Description:

Objectives:

We have previously shown in the guinea pig that a subpopulation of thymus derived or T-lymphocytes have the capacity to bind aggregated γ-globulin, a property hitherto believed to be exclusive to bone-marrow derived or B-cells. The findings suggested that T cells developed an Fc receptor upon activation. We aim to extend these findings to determine whether T cells (in man) develop an Fc receptor upon allogeneic stimulation in vitro. Furthermore we will compare these findings with T cells stimulated by mitogens.

Methods Employed:

Mixed lymphocyte reactions using human peripheral blood lymphocytes were set up using standard procedures. Aggregated guinea pig γ-globulin was used to detect the putative Fc receptor and simultaneous sheep cell rosettes used as a marker for T cells.

Major Findings:

Preliminary findings indicate that allogeneically stimulated T cells develop the capacity to bind aggregated γ-globulin in vitro and hence presumably acquire Fc receptors. Preliminary evidence showed that mitogen stimulated cells appeared to differ quantitatively with regard to Fc binding.

Significance to Bio-Medical Research and the Program of the Institute:

Fc receptors on T cells may well play a major role in basic immunological reactions such as for example antigen processing. Furthermore, reports of the existence of T cell immunoglobulin may be explained by adherence of exogenous Ig via this receptor. The finding of T cells with apparent Fc receptors in the MLC reaction may extend these findings to an in vitro model in man and provide a simple system for further characterization of this receptor.

Proposed Course:

Using the model systems described we will attempt to provide answers to the questions (1) do Fc receptors bearing T cells fall into one of the recently described functional categories of T cells--helper, suppressor, cytotoxic, (2) do anti-Ia antisera block T cell Fc receptors as has been shown for B cells?

Publications:

1. Manuscript in preparation.

| SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space) | U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT** | PROJECT NUMBER Z01 AM 41016-05 ARB |
|---|---|---|

PERIOD COVERED

July 1, 1976 to September 30, 1977

TITLE OF PROJECT (80 characters or less)

The removal of bilirubin and other albumin bound substances from blood

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

| | | | |
|---|---|---|---|
| PI: | P.H. Plotz | Senior Investigator | ARB NIAMDD |
| OTHER: | N. Catterall | Technician | ARB NIAMDD |
| | P. Berk | Chief, Section on Diseases of the Liver | DD NIAMDD |
| | B. Blitzer | Clinical Associate | DD NIAMDD |
| | J. Waggoner | Chemist | DD NIAMDD |

COOPERATING UNITS (if any)

Digestive Diseases Branch, NIAMDD

LAB/BRANCH

Arthritis and Rheumatism Branch

SECTION

Connective Tissue Disease

INSTITUTE AND LOCATION

NIAMDD, NIH, Bethesda, Maryland 20014

| TOTAL MANYEARS: | PROFESSIONAL: | OTHER: |
|---|---|---|
| 1 3/4 | 1¼ | ½ |

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Previous studies have established the feasibility of removing albumin-bound substances such as bilirubin from the blood by in vivo extracorporeal perfusion using albumin-agarose beads. Because a number of substances in addition to bilirubin accumulate in the blood of patients with liver damage, we plan to test albumin-agarose beads and other adsorbents in extracorporeal perfusion of experimental animals with liver damage. At present we are setting up the assays to measure blood levels of some substances known to accumulate in liver failure, amino acids, fatty acids, mercaptans, and the false neurotransmitter, octopamine. We will test the efficacy of various adsorbents for binding these substances.

6311

PHS-6040
(Rev. 10-76)

Project Description:

Objectives:

Substances such as bilirubin that bind tightly to plasma protein cannot be removed by dialysis. We have attempted to remove such substances by employing affinity chromatography on albumin-agarose beads.

Methods Employed:

Human albumin is conjugated to agarose: the resulting beads are placed in a column and plasma or whole blood, are passed over the column. Bilirubin or other substances are assayed before and after passage. The bound material is eluted and assayed.

Major Findings:

Agarose-albumin columns can bind in excess of 150 μg bilirubin per gram of wet weight of gel. The BR can be completely eluted and the gel reused. The gel material is compatible with blood and can be used in a perfusion system with living rats and newborn monkeys. Binding of calcium by citrate prevents platelet and white cell losses in the extracorporeal circuit. During the past year, studies were carried out on the binding to albumin-agarose various fatty acids important in hepatic failure. Unfortunately difficulties with the GCC apparatus limited the amount of data generated. The technician responsible for this work left the National Institutes of Health early in 1977.

Significance to Bio-Medical Research and the Program of the Institute:

Such conjugates may be useful for removing bilirubin and other protein-bound toxic substances from human blood.

Proposed Course:

No further work is planned this year.

Publications:

1. Wolkoff, A.W., Scharschmidt, B.F., Plotz, P.H. and Berk, P.D.: Purification of conjugated bilirubin: A new approach utilizing albumin-agarose gel affinity chromatography. Proc. Soc. Ex. Bio. Med. 152:20-23, 1976.

2. Scharschmidt, B.F., Martin, J.F., Shapiro, L.J., Plotz, P.H. and Berk, P.D.: Hemoperfusion through albumin-conjugated agarose gel for the treatment of neonatal jaundice in premature rhesus monkeys. J. Lab. Clin. Med. (In press).

| SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space) | U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT** | PROJECT NUMBER Z01 AM 41018-03 ARB |
|---|---|---|

PERIOD COVERED
July 1, 1976 to September 30, 1977

TITLE OF PROJECT (80 characters or less)

Production of covalently-crosslinked immune complexes

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

| | | | |
|---|---|---|---|
| PI: | Paul H. Plotz | Senior Investigator | ARB NIAMDD |
| OTHER: | Ruth Guyer | Staff Fellow | ARB NIAMDD |
| | Robert Kimberly | Clinical Associate | ARB NIAMDD |
| | David Segal | Senior Investigator | IB NCI |

COOPERATING UNITS (if any)

Immunology Branch, National Cancer Institute

LAB/BRANCH
Arthritis and Rheumatism Branch

SECTION
Connective Tissue Disease

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20014

| TOTAL MANYEARS: | PROFESSIONAL: | OTHER: |
|---|---|---|
| 2 | 1½ | ½ |

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Antibodies have been site-specifically covalently cross-linked with a newly synthesized class of compounds which act as bis-affinity-labeling hapten. These oligomers of IgG are used as model immune complexes. They are separated by size and the biological properties of the sized oligomers are studied. Properties presently under study include complement fixation and Clq binding and biological survival.



PHS-6040
(Rev. 10-76)

Project Description:

Objectives:

To study the role of the configuration of immune complexes in determining their pathogenetic properties.

Methods Employed:

Anti-hapten antibodies are site-specifically cross-linked and the resulting oligomers are separated by size. Biological survival and complement fixation are studied in the standard fashion.

Major Findings:

Oligomers are removed from circulation with a rate inversely proportional to their size. Rate of removal is not apparently related to complement.

Significance to Bio-Medical Research and the Program of the Institute:

This work may allow studies of the properties of stable immune complexes of various sizes relevant to their role in the pathogenesis of connective tissue disease.

Proposed Course:

These model complexes will be used to probe the function of the RE system (i.e. biological survival of complexes) in a variety of states in which immune complex handling may be altered.

Publications:

1. Plotz, PH.: Bivalent affinity-labeling haptens in the formation of model immune complexes. Methods in Enzymology 46:505-508, 1977.

| SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space) | U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT** | PROJECT NUMBER Z01 AM 41020-10 ARB |
|---|---|---|

PERIOD COVERED

July 1, 1976 to September 30, 1977

TITLE OF PROJECT (80 characters or less)

Pathogenesis of Autoimmunity in New Zealand Mice

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

| | | | |
|---|---|---|---|
| PI: | A.D. Steinberg | Senior Investigator | ARB NIAMDD |
| OTHER: | L. Klassen | Clinical Associate | ARB NIAMDD |
| | G. Williams | Clinical Associate | ARB NIAMDD |
| | J. Reinertsen | Clinical Associate | ARB NIAMDD |
| | J. Reeves | Chemist | ARB NIAMDD |
| | E. Raveche | Chemist | LEP NIAMDD |
| | R. Skelly | Guest Worker | Rutgers Univ. |
| | R. Krakauer | | Div. Rheum. Mass. Gen. Hospital |
| | M. Gelfand | | Georgetown Univ. Hosp. |

COOPERATING UNITS (if any)

Laboratory of Experimental Pathology, NIAMDD
Rutgers University
Georgetown University Hospital

LAB/BRANCH

Arthritis and Rheumatism Branch

SECTION

Connective Tissue Disease

INSTITUTE AND LOCATION

NIAMDD, NIH, Bethesda, Maryland 20014

| TOTAL MANYEARS: | PROFESSIONAL: | OTHER: |
|---|---|---|
| 2 1/4 | 1 3/4 | ½ |

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☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The pathogenesis of autoimmunity in New Zealand mice is still uncertain; however, advances are being made. Genetic, viral and immunologic factors appear to be involved. The immunologic factors are complex. A very early defect is a loss of thymic regulatory or suppressor cells. These cells may keep the immune system in check and when deficient allow autoimmune responses to occur. We are trying to separate and characterize these cells with an aim toward understanding the precise cellular defect.

Antibodies to T cells are produced by New Zealand mice. The role of these antibodies in the loss of suppressor cells is being evaluated by several techniques. Administration of these anti-T cell antibodies to NZB/NZW mice from 3 days to 1 month of age preferentially eliminates suppressor cells.



PHS-6040
(Rev. 10-76)

Project Description:

Objectives:

New Zealand mice spontaneously develop an autoimmune disease resembling human systemic lupus erythematosus. Early in life they become relatively resistant to tolerance induction, later they spontaneously develop antibodies to RNA and DNA and finally they develop immune complex glomerulonephritis from which they die. Both humoral and cellular immune defects have been reported and are under active investigation.

Both helper and suppressor thymus derived (T) cells regulate antibody production by bone marrow derived (B) cells. Two distinct T cell subpopulations help or suppress cell mediated immunity. The existence of suppressor T cells has been supported by experiments in several systems. We have been investigating such suppressor cell function in New Zealand mice in the hope of understanding the immunologic basis of this autoimmune disease.

Methods Employed:

1. Assay for antibodies to RNA and DNA using $^{14}C$-labeled nucleic acids as ligands and the ammonium sulfate precipitation assay. Assay of antibody forming cells to sheep erythrocytes and pneumococcal polysaccharide by local hemolysis in gel.

2. Immunization of animals with a variety of antigens with and without protein carriers or adjuvants.

3. Transfer of spleen, bone marrow and thymus cells into recipients to evaluate the functional properties of the different cell types.

4. Use of standard skin grafting using allogenic skin to evaluate cellular immune functions in New Zealand mice, and transfer of serum and cell population between young and old New Zealand mice.

5. Study of graft versus host disease inducing properties of NZB/W lymphoid cells using the Simonsen spleen weight assay and newborn C3H/He or SWISS mice as recipients, or adult athymic (nude) mice.

6. Separation of lymphoid cells functionally (corticosteroid, ATS, anti-θ, anti-kappa) and physically (sedimentation).

7. Assay of anti-T cell antibodies by release of $^{51}Cr$ from thymocytes.

8. Evaluation of subpopulation of T cells using the fluorescence activated cell sorter.

9. Castration of males and female weanling NZB/W mice and treatment with sex hormones by either injection or implantation of sialastic tubes containing crystaline hormones.

Major Findings:

1. NZB hybrids demonstrate a marked sex difference in spontaneous auto-antibody production: Females produce auto-antibodies earlier and at higher titers than males. Castration, but not vasectomy, of NZB x DBA/2 males increased NTA antibody production to the levels seen in females. Castration of NZB/NZW males accelerates their disease which is prevented by testosterne but not estrodiol. Castration of females by itself has little effect; however, castration plus testosterne retards disease.

2. Treatment of NZB/NZW females with nafoxidine, an estrogen inhibitor which acts by binding avidly to nuclear estrogen receptors, led to significant reduction in antibodies to DNA, reduced proteinuria and prolonged survival.

3. Treatment of baby NZB/NZW mice (3-5d of age) with naturally occuring anti-T-cell antibodies (NTA) led to premature loss of suppressor cells, accelerated autoimmunity and early death.

4. Injection of sheep erythrocytes into mice leads to a rise in splenic cyclic AMP with a peak at 2 minutes. This antigen-specific response is present in young NZB/NZW mice but not in old NZB/NZW mice. Splenic T cells from young NZB/NZW mice restores the response of old mice to normal.

5. Suppressor factors are produced by spleen culture from 2-week-old mice of all strains. Such factors are made by normal mice 6, 10 and 16 weeks of age, but not by NZB/NZW mice at these ages. The factor(s) are of low molecular weight (<10,000 daltons).

Proposed Course:

Future studies will attempt to further define and separate different subpopulations of T cells, as well as the relative changes in such populations with age (helper, suppressor, effector, etc.). The characteristics of the separated subpopulations will be defined, particularly those of suppressor cells. The role of genetic and viral factors and anti-T cell antibodies in the loss of suppressor cells will be further investigated in New Zealand mice. The availability of athymic nude mice will allow us to study lack of thymic function in different strains with and without superinfection with different viruses or immunization with nucleic acid antigens. We hope thereby to define the relative contributions of genetic, viral, and immunologic factors in the development of autoimmunity.

Significance to Bio-Medical Research and the Program of the Institute:

1. The role of sex hormones in modulating auto-antibody production provides a means by which disease modulation may be better understood, investigated and perhaps altered.

2. The role of anti-T cell antibodies in accelerating suppressor cell loss identifies an important pathogenetic mechanism of immune dysregulation.

3. Studies of cAMP in NZB/NZW mice following antigen administration begins to develop understanding of immune effects and their abnormalities at a biochemical level. The cAMP response to antigen is most likely a signal to turn off or suppress other immune responses. No such signal is generated in the older autoimmune NZB/NZW mice who behave as if they are already maximally stimulated antigenically.

4. Studies of suppressor factors extend our understanding of antigen non-specific suppression and augmentation in autoimmunity. First there is a loss of suppressor signals which is followed by the development of autoimmunity and a secondary increase in suppressor signals which are insufficient to counteract the autoimmune process.

Publications:

1. Raveche, E.S., Klassen, L.W. and Steinberg, A.D.: Sex differences in the formation of anti-T-cell antibodies. Nature 263:415-416, 1976.

2. Eilat, D., Schechter, A.N. and Steinberg, A.D.: Antibodies to native tRNA in NZB/NZW mice. Nature 259:141-143, 1976.

3. Gershwin, M.E., Steinberg, A.D., Ahmed, A. and Derkay, C.: Studies of thymic factors. II. Failure of thymosin to alter the natural history of New Zealand mice. Arth. Rheum. 19:862-866, 1976.

4. Ranney, D.F. and Steinberg, A.D.: Differences in the age-dependent release of a low molecular weight suppressor (LMWS) and stimulators by normal and NZB/W lymphoid organs. J. Immunol. 117:1219-1225, 1976.

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6. Gershwin, M.E. and Steinberg, A.D.; The pathogenetic basis of animal and human autoimmune disease. Seminars in Arth. Rheum. 6:125-165, 1976.

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| SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space) | U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT** | PROJECT NUMBER Z01 AM 41021-09 ARB |
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PERIOD COVERED

July 1, 1976 to September 30, 1977

TITLE OF PROJECT (80 characters or less)

Antinuclear antibodies in spontaneous and drug-induced systemic lupus erythematosus and other diseases

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

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| OTHER: | D. Eilat | Visiting Scientist | LCB NIAMDD |
| | M. Akizuki | Visiting Fellow | ARB NIAMDD |
| | E.S. Raveche | Chemist | LEP NIAMDD |
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COOPERATING UNITS (if any)

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LAB/BRANCH

Arthritis and Rheumatism Branch

SECTION

Connective Tissue Disease

INSTITUTE AND LOCATION

NIAMDD, NIH, Bethesda, Maryland 20014

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|---|---|---|
| 2 | 1 3/4 | 1/4 |

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Antibodies to nucleic acids are important diagnostically and pathogenetically in systemic lupus. The production of these antibodies is in part controlled by x-linked immune response genes. In addition, sex hormones influence the magnitude of auto-antibody production. Antibodies to transfer RNA are produced by patients with SLE; these cross react with viral nucleic acids. Subpopulations of patients have specific auto-antibodies.



PHS-6040
(Rev. 10-76)

Project Description:

Objectives:

Study the characteristics of a variety of antinuclear antibodies in human and murine "autoimmune diseases." Determine genetic contributions to the immune response to nucleic acid and related antigens.

Methods Employed:

Farr ammonium sulfate precipitation assay using radiolabelled ligands. Standard precipitin reaction using labeled and unlabeled nucleic acids or nuclear extracts. Hemagglutination, complement fixation and immunodiffusion assays. Standard immunization procedures.

Major Findings:

1. Some strains of mice (NZB and ALN) were found to have high responses to immunization with single stranded DNA complexed to a carrier protein. These animals also spontaneously develop antinuclear antibodies late in life. In contrast DBA/2 and BALB/C mice responded poorly to immunization with SSDNA. These mice do not spontaneously develop antinuclear antibodies. Genetic studies of NZB x DBA $F_1$, mice and backcrosses to the parental strains suggest that a gene on the X chromosome may influence the response to immunization with SS DNA. Quantitatively, much of this response is regulated by sex hormones (sec. 2).

2. Castration of male NZB x DBA or DBA x NZB mice led to accelerated anti-DNA production, both following immunization and spontaneously.

3. Patients with a) myasthenia gravis or b) IgA deficiency have an increase in antibodies to nucleic acids.

4. Sioux, Crow and Arapaho North American Indians have an increase in antinuclear antibodies in association with an increased incidence of SLE.

5. A radioimmunoassay for a soluble nuclear antigen has been developed. Antibodies are found in patients with Sjogren's syndrome without associated connective tissue disease (sicca complex) or Sjogren's syndrome with associated SLE. Patients with Sjogren's syndrome and rheumatoid arthritis are negative as are patients without Sjogren's syndrome.

Significance to Bio-Medical Research and the Program of the Institute:

X-linked immune response genese help to control the immune responses to nucleic acid antigens. Male sex hormones appear to suppress auto-antibody production explaining the more severe disease and greater incidence in females. There is a greater effect on immune complex deposits than on auto-antibody production. In contrast, prevention of action of female sex hormones has a greater impact on decreasing anti-DNA production.

The finding of subpopulations of humans with specific auto-antibodies or with increased incidence of autoimmunity allows for further study to dissect the basic abnormalities in the population.

Proposed Course:

Define the genetics of the antibody response to DNA. Further study the characteristics of antinuclear antibodies. Continue to evaluate the role of sex hormones in autoimmunity. Compare antinuclear antibodies of patients with different autoimmune diseases (SLE, Sjogren's syndrome, rheumatoid arthritis). Further study subpopulations of patients with a high incidence of autoimmunity

Publications:

1. Scher, I., Ahmed, A., Sharrow, S.P., Steinberg, A.D. and Paul, W.E.: Genetic control of B-lymphocyte function in the CBA/N mouse strains. A model for examining the mechanism of B-lymphocyte activation. In Mitogens in Immunobiology. J.J. Oppenhiem and D.L. Rosenstreich, (eds.) Academic Press, 1976 pp. 325-335.

2. Gershwin, M.E., Glinski, W., Bender, A.N., Ringel, S.P., Steinberg, A.D. and Engel, W.K.: Antibodies to nucleic acids in myasthenia gravis. Int. Arch. Allergy and Appl. Immunol. 51:245-252, 1976.

3. Gershwin, M.E., Blaese, R.M., Steinberg, A.D., Wistar, R., Jr. and Strober, W.: Antibodies to nucleic acids in association with IgA deficiency. J. Pediatrics 89:377-387, 1976.

4. Morton, R.O., Gershwin, M.E., Brady, C. and Steinberg, A.D.: Incidence of systemic lupus erythematosus (SLE) in North American Indians. J. Rheumatol. 3:186-190, 1976.

5. Kassen, S.S., Akizuki, M., Steinberg, A.D., Reddick, R.L. and Chused, T.M.: Antibody to a soluble acidic nuclear antigen in Sjogren's syndrome. Amer. J. Med. (In press).

6. Gershwin, M.E., Merchant, B. and Steinberg, A.D.: The effects of synthetic polymeric agents on immune responses of nude mice. Immunol. 32:327-336, 1977.

7. Gershwin, M.E., Glinski, W., Chused, T.M. and Steinberg, A.D.: Antibody dependent lymphocyte mediated cytolysis of DNA-anti-DNA coated target cells using human and murine effector populations: A possible mechanism of immunopathology in systemic lupus erythematosus. Immunol. (In press).

| SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space) | U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT** | PROJECT NUMBER Z01 AM 41022-06 ARB |
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PERIOD COVERED

July 1, 1976 to September 30, 1977

TITLE OF PROJECT (80 characters or less)

Therapeutic studies in New Zealand mice

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

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Division of Rheumatology, Massachusettes General Hospital, Boston, Mass.

LAB/BRANCH

Arthritis and Rheumatism Branch

SECTION

Connective Tissue Diseases

INSTITUTE AND LOCATION

NIAMDD, NIH, Bethesda,Maryland 20014

| TOTAL MANYEARS: | PROFESSIONAL: | OTHER: |
|---|---|---|
| 2 1/4 | 1 3/4 | ½ |

CHECK APPROPRIATE BOX(ES)

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Non-specific immunosuppressive therapy of man is associated with considerable morbidity. Some regimens are superior to others. The New Zealand mice offer a convenient model for studying different drug regimens prior to study in patients.

It would be even more desirable to treat patients in an immunologically more specific manner. Toward that end we have been treating New Zealand mice with activators of suppressor cells and with suppressor cells themselves. The autoimmune process in NZB mice can be markedly retarded by such specific immunosuppression. Soluble suppressor substances have been substituted for cells and found to be effective. If such a substance can be isolated and synthesized it might be useful for humans.

Treatment of NZB/NZW mice with Ribavirin, a broad spectrum anti-viral drug, has been very effective. Renal deposits of immune complexes can be prevented. Such treatment is applicable to humans with SLE

Project Description:

Objectives:

NZB/NZW $F_1$ mice spontaneously develop antibodies to nucleic acids and die of immune complex glomerulonephritis. NZB mice develop Coomb's positive hemolytic anemia, membranous nephrosis and excessive lymphoreticular proliferation. These mice are an excellent model for comparing different immunosuppressive and anti-viral drug programs. In addition, as the immunologic basis for the disease becomes better understood, they offer a model for studying more specific immunotherapy.

Methods Employed:

1. Comparison of different immunosuppressive and anti-viral drug regimens in natural history of NZB/NZW mice by study of antibodies to DNA, proteinuria and survival.

2. Treatment with suppressor cells and/or suppressor substances derived from such cells in an attempt at restoring normal regulatory functions and improve the natural history of New Zealand mice.

Major Findings:

1. Ribavirin, a broad spectrum anti-viral agent, was found to be effective (a) prophylactically and (b) therapeutically in NZB/NZW mice. The favorable effects included: decreased proteinuria, decreased anti-DNA antibodies, and increased survival. No toxicity was observed. Little immunosuppression was seen. Ribavirin was at least as effective as cyclophosphamide.

2. Suppressor cells from young NZB mice were given to NZB mice from 4 weeks of age with retardation in (a) autoimmune hemolytic anemia (b) renal disease (c) lymphoreticular proliferation. The relevant cell was corticosteroid and X-radiation sensitive.

3. Supernatants from BALB/c spleen cells stimulated with Concanavalin A were given to NZB/NZW mice starting at 18-19 weeks of age. Such mice had prolonged survival. The <10,000 molecular weight fraction was at least as effective as the whole supernatant.

Significance to Bio-Medical Reserach and the Program of the Institute:

These studies are designed to provide background experience for clinical studies in which drugs may be given in a more effective manner to humans with SLE. Trials of the anti-viral drug Ribavirin will be considered in humans on the basis of these studies.

Continued study in mice may provide the background information necessary

for immunological intervention in humans with SLE. Suppressor factors may be of value in humans in the future.

Proposed Course:

Further study of anti-viral agents. Attempt to better treat New Zealand mice with soluble suppressor substances and to better characterize such substances. Immunologic studies with suppressor substances.

Publications:

1. Steinberg, A.D.: Clinical use of immunoregulants. In Immunopharmacology, M.E. Rosenthale and H.C. Mansmann, Jr. (eds.), Spectrum Publications, New York 1976, pp. 267-274.

2. Morton, R.O., Goodman, D.G., Gershwin, M.E., Derkay, C., Squire, R. A. and Steinberg, A.D.: Suppression of autoimmunity and lymphoid proliferation in NZB mice with steroid-sensitive X-radiation sensitive syngeneic young thymocytes. Arth. Rheum. 19:1347-1350, 1976.

3. Klassen, L.W., Budman, D.R., Williams, G.W., Steinberg, A.D. and Gerber, N.L.: Antiviral agents in treatment of murine autoimmune disease. Science 195:787-789, 1977.

4. Steinberg, A.D. and Klassen, L.W.: Role of suppressor T cells in lymphopoietic disorders. Clinics in Haematology 6:439-478, 1977.

5. Gerber, N.L., Powell, D. and Steinberg, A.D.: Therapeutic studies in NZB/NZW $F_1$ mice. V. Comparison of cyclophosphamide and chlorambucil. Arth. Rheum. ( In press, 1977).

6. Reinertsen, J.L. and Steinberg, A.D.: In vivo immune response suppression by the supernatant from Concanavalin A - activated spleen cells. J. Immunol. 119:217-222, 1977.

| SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space) | U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT** | PROJECT NUMBER Z01 AM 41023-03 ARB |
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PERIOD COVERED

July 1, 1976 to September 30, 1977

TITLE OF PROJECT (80 characters or less)

Study of Immune Abnormalities in Patients with Systemic Lupus Erythematosus (SLE)

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

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| | L. Klassen | Clinical Associate | ARB NIAMDD |
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| | W. Glinski | Guest Worker | DB NCI |
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| | I. Green | Senior Investigator | LCI NIAMDD |
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| | T. Waldmann | Chief, Metabolism Br. | MB NCI |
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Bureau of Biologics
Laboratory of Clinical Investigations, NIAMDD
Metabolism Branch, NCI
Immunology Branch, NCI

LAB/BRANCH

Arthritis and Rheuamtism Branch

SECTION

Connective Tissue Disease

INSTITUTE AND LOCATION

NIAMDD, NIH, Bethesda, Maryland 20014

| TOTAL MANYEARS: | PROFESSIONAL: | OTHER: |
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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Patients with SLE have anti-T-cell antibodies and loss of a subpopulation of T cells. This subpopulation regulates B cell activity. In the absence of adequate regulatory function, excessive antibody forming cells are found. These cells have specificity for a variety of chemical haptens rather than just auto-antigens. Excessive B cell activity and loss of regulatory cells occurs in patients with active SLE, and is often normal in SLE patients in remission. Loss of both helper T cells and suppressor T cells may occur in patients with active SLE.

Project Description:

Objectives:

To apply the newer understanding of basic immunologic principles and observations in New Zealand mice (who develop a lupus-like disease) to the study of patients with SLE.

Methods Employed:

1. Study of families of patients with SLE or larger groups of patients with a high incidence of SLE. A battery of immunologic measurements would be made in such patients.

2. Study of lymphocytes from patients with SLE.
   a. Distribution on discontinous gradient.

   b. Number and distribution of T, B and null cells and their responses to mitogens or allogenic cells.

   c. Relationship between anti-lymphocyte antibodies in patients with SLE and abnormalities of cell number and/or function.

   d. Study of antibody producing plague forming cells (PFC) in the peripheral blood of patients.

   e. Study of anti-lymphocyte antibodies by use of the fluorescence activated cell sorter (FACS).

Major Findings:

1. Patients with SLE have abnormal distributions of peripheral blood lymphocytes. Those with active disease have a relative decrease in a subpopulation of T cells and an increase in null cells.

2. Anti-lymphocytes antibodies occur in association with increased activity of patients with SLE. These antibodies can reduce the number of peripheral T cells and interfere with their function. Both helper and suppressor cells are deficient in patients with active SLE.

3. Antibodies to human lymphocytes found in sera from patients with SLE are readily studied on the FACS. IgM antibodies to T cells recognize 100% of the T cells of normals and patients with SLE, indicating no specificity for a subpopulation - such as helper or suppressor cells.

4. Purified T cell populations were activated with Concanavalin A and studied for ability to suppress B and T cell functions. SLE patient's T cells were defective in their ability to generate suppressor signals, but were able to respond to normal suppressor signals.

5. Patients with active SLE have increased quantities of antibody producing PFC to defined chemical haptens. Patients with inactive SLE were not significantly different from controls.

6. In a randomized double-blind trial of influenza vaccination in 40 SLE patients, those receiving influenza vaccine had no increase in adverse effects.

Significance to Bio-Medical Research and the Program of the Institute:

Populations for further study are being defined and techniques refined for continued study of humans with SLE. It is hoped that the rewarding observations in the animal model of SLE, New Zealand mice, can continue to be applied to the study of the human disease itself.

The studies of PFC suggest that patients with SLE have generalized B cell hyperactivity rather than selective B cell hyperactivity towards self-antigens. Loss of suppressor and helper cells in human SLE is associated with (a) disease activity and (b) anti-T-cell antibodies suggests that the lesion is not permanent and that appropriate specific prevention may be possible.

The ability of SLE patients to tolerate influenza vaccination without flares strikes down anecdotes in the literature regarding the undesirability of immunizing SLE patients. This will allow such people to receive adequate vaccination programs in the future.

Proposed Course:

Continue the studies initiated.

Publications:

1. Glinski, W., Gershwin, M.E., Budman, D.R. and Steinberg, A.D.: Study of lymphocyte subpopulations in normal humans and patients with systemic lupus erythematosus by fractionation of peripheral blood lymphocytes on a discontinuous ficoll gradient. Clin. Exp. Immunol. 26:228-238, 1976.

2. Budman, D.R. and Steinberg, A.D.: Current concepts of the hematologic aspects of systemic lupus erythematosus. Ann. Intern. Med. 86:220-229, 1977.

3. Budman, D.R., Merchant, E.B., Steinberg, A.D., Doft, B., Gershwin, M. E., Lizzio, E. and Reeves, J.P.: Increased spontaneous activity of antibody forming cells in the peripheral blood of patients with active systemic lupus erythematosus. Arth. Rheum. 20:829-833, 1977.

| SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space) | U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT** | PROJECT NUMBER Z01 AM 41024-10 ARB |
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PERIOD COVERED
July 16, 1976 to September 30, 1977

TITLE OF PROJECT (80 characters or less)

The Biochemical Lesions in the Genetic Mucopolysaccharidoses and Mucolipidoses

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

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| | Clara W. Hall | Research Chemist | ARB | NIAMDD |
| | Ingeborg Liebaers | Visiting Associate | ARB | NIAMDD |
| | Ngozi Nwokoro | Visiting Fellow | ARB | NIAMDD |
| | Leonard Rome | Staff Fellow | ARB | NIAMDD |
| | Gloria Sando | Guest Worker | ARB | NIAMDD |
| | James Scott | Biologist | ARB | NIAMDD |
| | Ake Wasteson | Guest Worker | ARB | NIAMDD |

COOPERATING UNITS (if any)

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Arthritis and Rheumatism Branch

SECTION
Human Biochemical Genetics

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20014

| TOTAL MANYEARS: | PROFESSIONAL: | OTHER: |
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| 11 | 10 | 1 |

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The pinocytosis of the lysosomal enzyme, α-L-iduronidase into cultured fibroblasts is a receptor-mediated process apparently requiring a phosphorylated sugar on the enzyme. The biosynthesis of that marker in normal cells from patients with Mucolipidoses II and III will be examined. The role of secretion and pinocytosis in the transport of enzymes to lysosomes will be investigated.

A micro-assay for iduronate sulfatase in hair follicles may provide the basis for detecting heterozygotes of the Hunter syndrome. Purification of that enzyme from plasma has been undertaken in the hope of preparing therapeutically useful concentrates.

Project Description:

Objectives:

To study normal lysosomal function as well as the biochemical defects in genetic disorders of such function, as in mucopolysaccharidoses and mucolipidoses, and to use the findings for medical assistance to the patients and their families.

Methods Employed:

Standard methods of enzymology are used to assay and purify α-L-iduronidase and iduronate sulfatase from human tissues and body fluids. Skin fibroblasts are cultured by conventional methods and the kinetics of lysosomal enzyme are measured.

Major Findings:

1. Iduronidase has been purified 20,000 fold from human kidneys, to approximately 80% homogeneity. The enzyme is a glycoprotein of 63,000 molecular weight, separable into two subunits of equal size. Its amino acid composition and some kinetic properties have been determined.

2. One form of iduronidase, a "high uptake" form, is taken into fibroblasts by a receptor-mediated pinocytosis, as evidenced by Michaelis-Menten kinetics of uptake. The $K_{uptake}$ is $10^{-9}$ M. Uptake is inhibited competitively by a urinary glycoprotein fraction, with a $K_i$ less than $10^{-8}$ M, as well as by mannose 6-phosphate, with $K_i$ of $5 \times 10^{-5}$ M. Except for fructose 1-phosphate, no phosphorylated compound tested showed such inhibition. The structural feature on the lysosomal enzyme responsible for uptake is therefore thought to be a phosphorylated sugar, perhaps mannose 6-phosphate or a steric analog.

3. A number of procedures for detecting Hunter heterozygotes have been tried. Present tests include assays of uncloned fibroblasts or on lymphocytes, which were found unreliable, or of cloned fibroblasts, which are reliable, but too laborious to be practical. A micro-assay for iduronate sulfatase in hair follicles may provide a practical test; the follicles are nearly clonal in origin, and we have been able to find both active and inactive follicles in each of the known heterozygotes tested, in accordance with the Lyon hypothesis.

4. The purification of iduronate sulfatase from plasma has been undertaken, and the enzyme purified about 1000 fold.

5. A procedure has been developed for examining the cells and tissues of patients with iduronidase deficiency diseases (i.e., Hurler and Scheie syndromes) for cross-reactive proteins. So far, none has been found in a very small number of cultures from Hurler patients that have been examined.

Significance to Bio-Medical Research and the Program of the Institute:

This project, which was started ten years ago, has led to accurate diagnosis, improved genetic counseling and prenatal diagnosis for the genetic diseases of mucopolysaccharide storage. The current development of heterozygote detection tests for the X-linked Hunter syndrome should provide a much-needed service to the women who know themselves to be at high-risk from family histories. Purification of plasma iduronate sulfatase will hopefully lead to the preparation of enzyme concentrates suitable for clinical trial of enzyme replacement therapy for the Hunter syndrome.

Studies of the secretion and uptake of lysosomal enzymes should be relevant to both normal (e.g., bone remodeling) and pathological (e.g., rheumatoid arthritis) conditions. In addition, the finding of specific receptors on fibroblasts for a phosphoglycoprotein may be ultimately useful for targeting liposomes (used to encapsulate toxic or highly radioactive drugs) to connective tissue.

Proposed Course:

1. Studies of the recognition marker and cell receptor will be expanded. Since attempts to prepare $^{125}$I-labeled high uptake enzyme have not been successful, an alternative approach will be to prepare synthetic ligands - e.g., by coupling mannose 6-phosphate or oligosaccharides containing terminal mannose 6-phosphate to albumin or Sepharose. (A modification might be to couple mannose residues and then phosphorylate them). These mannose 6-phosphate containing macromolecules will be labeled with radioiodine and used for receptor binding studies. If a suitable assay can be developed, purification of the receptor will be undertaken.

Another approach will be to label high uptake enzymes biosynthetically, using cultured cells incubated in the presence of radioactive phosphate and purifying the glycoproteins secreted into the medium.

2. This laboratory had previously proposed that the primary defect in mucolipidoses II and III was in some posttranslational modification of hydrolytic enzymes, so that they were devoid of a recognition marker and unable to reach their normal location within lysosomes. Now that the marker has been identified as a phosphorylated sugar, it is technically possible to determine whether it is missing or masked in the mucolipidoses, and to proceed systemically to find the basic defect in these disorders.

3. The purification of iduronate sulfatase from urine and plasma will be continued with the following goals: a) preparation of antibody and recognition of cross-reactive proteins in cells of Hunter patients, b) determination of uptake mechanism; c) preparation of plasma concentrates for trials of

enzyme replacement.

4. The life-cycle of a lysosomal enzyme will be studied by pulse-chasing the enzyme and following its path from the endoplasmic reticulum to the cell exterior and to lysosomes. Iduronidase will be the enzyme studied because of our extensive experience with that enzyme and because an antibody has been prepared for the purpose. The question to be answered is whether entry into lysosomes precedes or follows its appearance extracellularly.

Publications:

1. Liebaers, I. and Neufeld, E.F.: Iduronate sulfatase activity in serum, lymphocytes and fibroblasts - simplified diagnosis of the Hunter syndrome. Pediat. Res. 10: 733-736, 1976.

2. Liebaers, I., Di Natale, P., and Neufeld, E.F.: Iduronate sulfatase in amniotic fluid: an aid in the prenatal diagnosis of the Hunter syndrome. J. Pediat. 90: 423-425, 1977.

3. Epstein, C.J., Yatsiv, S., Neufeld, E.F., and Liebaers, I.: Genetic counseling for Hunter syndrome. Lancet II, 737, 1976.

4. Neufeld, E.F.: The enzymology of inherited mucopolysaccharide storage disors. Trends in Biochemical Sciences 2: 25-26, 1977.

5. Di Natale, P., Leder, I.G., and Neufeld, E.F.: A radioactive substrate and assay for α-L-iduronidase. Clin. Chim. Acta, in press.

6. Neufeld, E.F., Sando, G.N., Garvin, A.J., and Rome, L.H.: The transport of lysosomal enzymes. J. Supramol. Struct., in press.

7. Migeon, B.R., Sprenkle, J.A., Liebaers, I., Scott, J.F., and Neufeld, E.F.: X-linked Hunter syndrome: the heterozygous phenotype in cell culture. Am. J. Hum. Genet., in press.

8. Neufeld, E.F., Liebaers, I., Epstein, C.F., Yatsiv, S., Milunsky, A., and Migeon, B.R.: The Hunter syndrome in females: is there an autosomal recessive form of iduronate sulfatase deficiency: Am. J. Hum. Genet., in press.

| SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space) | U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT** | PROJECT NUMBER Z01 AM 41025-06 ARB |
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PERIOD COVERED
July 1, 1976 to September 30, 1977

TITLE OF PROJECT (80 characters or less)

Studies on the Cell Surface Receptor for IgE

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

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| | S. Newman | Staff Fellow | ARB NIAMDD |
| | G. Rossi | Visiting Scientist | ARB NIAMDD |
| | J. Kanellopoulos | Post-doctoral Fellow | ARB NIAMDD |
| | J. Taurog | Research Associate | ARB NIAMDD |
| | D. Segal | Senior Investigator | IB NCI |
| | R. Siraganian | Chief, Clin. Immunol. Sec. | LMI NIDR |
| | W. Hook | Senior Investigator | LMI NIDR |
| | J. Schlessinger | Post-doctoral Fellow | Dept. Chem. Cornell U |
| | E. Elson | Assistant Professor | Dept. Chem. Cornell U |
| | W. Webb | Professor of Physics | Dept. Phys. Cornell U |

COOPERATING UNITS (if any)

IB, NCI; LMI, NIDR; Dept. Chem, Cornell University

LAB/BRANCH
Arthritis and Rheumatism

SECTION
Chemical Immunology

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20014

| TOTAL MANYEARS: | PROFESSIONAL: | OTHER: |
|---|---|---|
| 8½ | 6 | 2½ |

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

We are pursuing an in-depth investigation on the structure and function of the membrane receptor for IgE. For these studies intact cells from a rat basophilic leukemia and a mouse mastocytoma as well as normal rat and mouse mast cells are being used. In addition receptor-bearing membrane particles and solubilized receptors are being studied, with the aim being to understand the structure of the receptor as well as its integration in the membrane. Results of such studies as well as direct functional studies are aimed at understanding the early steps in the cellular triggering by an antigen-induced antibody-mediated reaction.

2. Studies of the solubilized receptors.

a. Molecular weight studies

Our newly developed assay method (3) was used to determine the molecular weight of the receptor in detergent by sedimentation-diffusion analysis (4). The data yielded the following estimates (all based on direct measurements of the species shown by the same method).

| | |
|---|---|
| IgE | 200,000 |
| Receptor | 130,000 |
| IgE-Receptor Complex | 310,000 |

Although the methods have a somewhat uncertain accuracy (despite their excellent precision) the excellent agreement of the value determined for IgE with its known size by other methods, as well as the consistency of the values with the previous data on the valency of the receptor (see above), gives us confidence that the numbers are substantially correct. Too many assumptions must be made to have great confidence in our estimate of the detergent-free molecular weight of the receptor. We are therefore using a different approach (below) in order to learn something about the polypeptide chain multiplicity of the receptor.

b. Purification of the receptor.

We are trying to develop a method by which the receptor can be purified in large amounts and relatively inexpensively. We hope to be able to use solid tumors rather than cultured cells for this purpose. Initial attempts to trap solubilized receptors on an IgE column and to then elute them with brief acidification did not prove successful - non-specifically adherant contaminants eluted with the receptor. The following protocol is beginning to yield promising results:

1. Homogenization of minced tumor in non-ionic detergent.

2. High speed centrifugation.

3. Addition of stoichiometric amounts of haptenated IgE to supernatant.

4. Prepurification of receptor-IgE complexes on lectin column -eluted with sugar.

5. Purification of receptor-IgE complexes on anti-hapten column - elution with hapten.

6. Trapping of complexes on anti-IgE beads - washing of beads to get rid of carrier protein.

7. Release of free receptor by reduction (and/or acid).

3. Studies on the mechanism of receptor action.

a. Use of anti-receptor antibodies

i. Immunization of rabbits with membrane fragments of rat basophilic leukemia cells led to the formation of anti-receptor antibodies. Evidence was obtained that antibodies to the combining site for IgE as well as to more distal portions of the receptor were elicited (5).

ii. New antisera prepared against pure (albeit denatured) receptors has been prepared which appears to be mono-specific. These antibodies will be used in an attempt to trigger histamine release directly. A positive result (which we anticipate) will a) demonstrate that the receptor for IgE is part of the signal generating mechanism (i.e. that it is a true receptor and not just an acceptor for IgE) and b) that the IgE plays only a passive role being used essentially as a mechanism by which antigen can induce receptor aggregation.

b. Minimal unit signal

It has been well documentated that aggregation of surface bound IgE is a necessary event in antigen (or in the laboratory, anti-IgE) mediated mast cell triggering. It has also been determined that the aggregation need not be extensive. We tried to define rigorously the minimal amount of aggregation necessary to generate a "unit signal." IgE was treated with the cross-linking reagent dimethyl suberimidate and separated the mixture into monomers, dimers, trimers etc. It was demonstrated unequivocally that dimers of IgE are sufficient to cause triggering (6).

c. Triggerable tumor

In order to progress in our understanding of IgE mediated triggering one will need large numbers of cells from which potential intermediates can be isolated and characterized. Normal cells are difficult to purify (except in the rat) and inconvenient to obtain in very large numbers (e.g. $\leq 10^6$ cells/rat). We have now identified a culturable mouse mastocytoma which appears to be fully triggerable (7). This may open a new phase of study of mast cell exocytosis.

4. Studies on IgE.

We are continuing to study ways be which we can cleave IgE in order to identify those portions of the molecule responsible for binding to the receptor. We have so far not been successful in isolating active fragments.

Project Description:

Objectives:

IgE is a unique class of immunoglobulin which plays a predominant role in the physiological events associated with a variety of allergic phenomena. The immunoglobulin becomes attached to the surface of basophils and mast cells and, upon exposure to the allergenic antigen, mediates the release of histamine and other vasoactive amines from the cells to which it is attached. Although there is an increasing amount of information concerning the biochemistry of this reaction almost nothing is known about how the IgE acts as the cell surface transducer. The present project is directed towards increasing our understanding of how the IgE interacts with mast cells. In addition we wish to understand the mechanism by which the ternary complex of antigen-IgE antibody--cell surface receptor triggers the degranulation of basophilic cells.

Methods Employed:

The principle new and unusual method used to study this system during the past year has been the method of photobleaching of fluorescence on single cells. Other methods include cross-linking of IgE with dimethylsuberimidate; $^{3}$H-serotonin uptake to study degranulation.

Major Findings:

Since our previous report the following has been accomplished:

1. Studies of the receptor on intact cells.

   a. Valency, receptor-receptor interactions

      The valence of the receptor with respect to IgE binding has been determined to be unity. Cells were incubated with a mixture of fluorescein- and rhodamine-conjugated IgE, treated with anti-fluorescein and examined for comigration of the two fluorochromes. Our failure to observe comigration shows that the receptors are univalent and unconnected to each other prior to aggregation (1).

   b. Mobility

      The mobility of the receptors under a variety of conditions was assessed using fluorochrome-conjugated IgE and the method of photobleaching of fluorescence on normal rat mast cells and rat basophilic leukemia cells. These studies confirmed the univalence and independent motion of the receptors. The studies also suggested that at least a portion of the receptors might have restricted mobility perhaps due to interaction with microfilaments (2).

Significance to Bio-Medical Research and the Program of the Institute:

The IgE mast cell system is of significance in the pathophysiology of allergy, parasitic infections, and inflammation. It is also an experimentally convenient system to study mechanisms of antigen-induced antibody-mediated cellular reactions in general. Finally it is an excellent model for the study of exocytosis. So far we are continuing to make good progress in the elucidation of the molecular mechanisms of this system. Knowledge of these may eventually permit rational intervention in situations where the system contributes to pathology.

Proposed Course:

1. Purification and characterization of receptor.

2. Use of cross-linking reagents to define chain multiplicity of receptor.

3. Search for changes in receptor associated with triggering using either isolated receptor or receptor incorporated into artificial membranes.

4. Further characterization of receptor integration in membrane.

5. Continued effort to define region on IgE responsible for binding.

6. Search for early biochemical events in triggering using tumor cells and normal cells.

Publications:

1. Mendoza, G.R. and Metzger, H.: Distribution and valency of receptor for IgE on rodent mast cells and related tumor cells. Nature 264: 548-550, 1976.

2. Schlessinger, J., Webb, W.W., Elson, E.L. and Metzger, H.: The lateral motion and valence of Fc receptors on rat peritoneal mast cells. Nature 264: 550-552, 1976.

3. Rossi, G., Newman, S.A. and Metzger, H.: Assay and partial characterization of the solubilized cell surface receptor for Immunolgobulin E. J. Biol. Chem. 252: 704-711, 1977.

4. Newman, S.A., Rossi, G. and Metzger, H.: Molecular weight and valence of the cell-surface receptor for Immunoglobulin E. Proc. Nat'l. Acad. Sci., U.S.A. 74: 869-872, 1977.

5. Isersky, C., Mendoza, G.R. and Metzger, H.: The binding of antibodies to the solubilized and membrane integrated mouse and rat receptor for IgE. J. Immunol. 1977 (In press).

6. Segal, D.M., Taurog, J.D. and Metzger, H.: Dimeric Immunoglobulin E serves as a unit signal for mast cell degranulation. Proc. Nat'l. Acad. Sci., U.S.A. (In press).

7. Taurog, J.D., Hook, W., Siraganian, R. and Metzger, H.: IgE mediated histamine and serotonin release from cultured mouse mastocytoma cells. Fed. Proc. 36: 1215, 1977.

Publications Previously Cited As "In Press":

1. Metzger, H., Budman, D. and Lucky, P.: Interaction of IgE with rat basophilic leukemia cells. V. Binding properties of cell free particles. Immunochem. 13: 417-423, 1976.

2. DeLisi, C. and Metzger, H.: Some physical chemical aspects of receptor-ligand interactions. Immunol. Communications 5: 417-436, 1976.

3. Halper, J. and Metzger, H.: The interaction of IgE with rat basophilic leukemia cells. VI. Inhibition by IgGa immune complexes. Immunochem. 13: 907-913, 1976.

4. Mendoza, G.R. and Metzger, H.: Disparity of IgE binding between normal and tumor mouse mast cells. J. Immunol. 117: 1573-1578, 1976.

| SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space) | U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT** | PROJECT NUMBER Z01 AM 41028-02 ARB |
|---|---|---|

PERIOD COVERED
July 1, 1976 to September 30, 1977

TITLE OF PROJECT (80 characters or less)
Renal effects of aspirin and other non-steroidal anti-inflammatory drugs in systemic lupus erythematosus (SLE)

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

| | | | |
|---|---|---|---|
| PI: | P.H. Plotz | Senior Investigator | ARB NIAMDD |
| OTHER: | R. Kimberly | Clinical Associate | ARB NIAMDD |
| | J.R. Gill, Jr. | Chief, Hypertension and Endocrin. Br. | EB NHLI |

COOPERATING UNITS (if any)
Hypertension and Endocrinology Branch, NHLI

LAB/BRANCH
Arthritis and Rheumatism Branch

SECTION
Connective Tissue Disease

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20014

| TOTAL MANYEARS: | PROFESSIONAL: | OTHER: |
|---|---|---|
| 3/4 | ½ | ¼ |

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Aspirin, an important drug in the therapy of many connective tissue diseases including systemic lupus erythematosus, has been found (Seaman, W.E. and Plotz, P.H., Arth. Rheum. 19:155-160, 1976) to raise serum creatinine and BUN and lower creatinine clearance when administered to some patients with SLE as well as to some normal volunteers. Although this effect appears to be rapidly and completely reversible, if it is unrecognized it may falsely suggest deteriorating renal function due to activity of the underlying diesease. Our studies have shown that this effect is related to renal blood flow and tubular function, and that is correlates with renal output of prostaglandin. Other drugs besides aspiring which inhibit prostaglandin synthetase also cause these effects.



PHS-6040
(Rev. 10-76)

Project Description:

Objectives:

To investigate the mechanism of the apparent alteration in renal function induced by prostaglandin-synthetase-inhibiting non-steroidal anti-inflammatory drugs.

Methods Employed:

In patients requiring aspirin therapy, careful studies of renal function are performed. In selected patients with systemic lupus erythematosus, studies include inulin and PAH clearances to determine GFR, RBF, and tubular function.

Major Findings:

Aspirin and other non-steroidal anti-inflammatory drugs in therapeutic doses produces an acute fall in creatinine clearance in some patients with systemic lupus and in some normals.

Significance to Bio-Medical Research and the Program of the Institute:

Recognition of these effects should prevent misinterpretation of alterations in renal function in patients taking drugs that influence renal prostaglandin synthesis.

Proposed Course:

Observations will continue to be made, but no further formal series are planned.

Publications:

1. Seaman, W.E. and Plotz, P.H.: Effect of aspirin of liver tests in patients with RA or SLE and in normal volunteers. Arth. Rheum. 19: 155-160, 1976.

2. Kimberly, R.P. and Plotz, P.H.: Aspirin-induced depression of renal function. New. Eng. J. Med. 296:418-424, 1977.

3. Kimberly, R.P., Bowden, R.E., Gill, J.R., Keiser, H.R. and Plotz, P.H.: Effects of aspirin on renal blood flow, glomerular filtration rate and urinary prostaglandins in systemic lupus erythematosus. Submitted for publication 1977.

| SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space) | U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT** | PROJECT NUMBER Z01 AM 41029-01 ARB |
|---|---|---|

PERIOD COVERED
July 1, 1976 to September 30, 1977

TITLE OF PROJECT (80 characters or less)

Development of computerized clinical database for systemic lupus erythematosus (SLE)

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

| | | | |
|---|---|---|---|
| PI: | J.H. Klippel | Senior Investigator | ARB NIAMDD |
| OTHER: | J.L. Decker | Chief, Arthritis and Rheumatism Br. | ARB NIAMDD |
| | E.K. Harris | Chief, Laboratory of Applied Studies | LAS DCRT |
| | G. Shakarji | | DMB DCRT |

COOPERATING UNITS (if any)
Laboratory of Applied Statistics, DCRT
Data Management Branch, DCRT

LAB/BRANCH
Arthritis and Rheumatism Branch

SECTION
Connective Tissue Disease

INSTITUTE AND LOCATION
NIAMDD, NIH Bethesda, Maryland 20014

| TOTAL MANYEARS: | PROFESSIONAL: | OTHER: |
|---|---|---|
| 1½ | 1¼ | ¼ |

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The evaluation of long term randomized therapeutic studies of SLE would be facilitated by the development of a comprehensive clinical database. In this project, an ongoing computerized storage and retrieval system will be used to monitor clinical and therapeutic information on SLE patients participating in studies of immunosuppression and diffuse glomerulonephritis.



PHS-6040
(Rev. 10-76)

Project Description:

Objectives:

Long term randomized trials comparing the efficacies of several different forms of immunosuppressive therapies and prednisone in the treatment of SLE diffuse glomerulonephritis have been in progress within our branch since 1970. These studies require careful monitoring of sequential observation of drug administration, clinical and laboratory studies and complications to determine the superiority of any single program, It is the objective of this project to develop a computerized data storage and retrieval system to monitor and evaluate these clinical trials.

Methods Employed:

A time oriented database will be used to record drug administration, clinical manifestations, studies of renal function, relevant laboratory studies and select complications of SLE patients participating in immunosuppressive studies. Information will be made current at the time of each inpatient, as well as outpatient, Clinical Center visits. The entry of data will be made from terminals located within the Clinical Center and datafile stored at DCRT.

Major Findings:

None at this time.

Significance to Bio-Medical Research and the Program of the Institute:

This project will benefit ARB clinical research activities by (1) facilitating the monitering and assessment of therapeutic studies of SLE, (2) permitting analysis of interrelationships of multiple variables in the course of SLE and (3) improvement of the care of SLE patients participating in ARB studies.

Proposed Course:

The programing phase of the project has been completed. The entry of data will be made both from retrospective information collected from patients involved in current trials and prospectively once a file has been established.

Publications:

None at this time.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE
PROJECT NUMBER (Do NOT use this space)

U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE
PUBLIC HEALTH SERVICE
NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 AM 41030-01 ARB

PERIOD COVERED

July 1, 1976 to September 30, 1977

TITLE OF PROJECT (80 characters or less)

Clearance of immune complexes by patients with systemic lupus erythematosus

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

| | | | |
|---|---|---|---|
| PI: | P.H. Plotz | Senior Investigator | ARB NIAMDD |
| OTHER: | R.P. Kimberly | Clinical Associate | ARB NIAMDD |
| | M. Frank | Chief, Lab. of Clin. Invest. | LCI NIAID |
| | T.J. Lawley | Investigator | DB NCI |

COOPERATING UNITS (if any)

Laboratory of Clinical Investigations, NIAID
Dermatology Branch, NCI

LAB/BRANCH

Arthritis and Rheumatism Branch

SECTION

Connective Tissue Disease

INSTITUTE AND LOCATION

NIAMDD, NIH, Bethesda, Maryland 20014

| TOTAL MANYEARS: | PROFESSIONAL: | OTHER: |
|---|---|---|
| 1 | 1/2 | ½ |

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Clearance of IgG and IgM-coated antologous red cells is studied in patients with systemic lupus not taking corticosteroids. Abnormal clearance of IgG-coated cells has been found in some patients with active disease.

Project Description:

Objectives:

To determine whether altered clearance of immune complexes is pathogenetic in systemic lupus

Methods Employed:

Autologous red cells are labeled with $^{51}Cr$ and coated with anti-blood group antibody. The cells are returned to the donor and samples drawn for several hours are tested for radioactivity.

Major Findings:

Clearance of IgG-coated RBC is abnormal in some patients with systemic lupus.

Significance to Bio-Medical Research and the Program of the Institute:

This method allows a probe of RE clearance of immune complexes in a disease in which the presence of complexes is probably pathogenetic.

Proposed Course:

Work will continue.

Publications:

1. Frank, M.M., Jaffe, C.J., Kimberly, R.P., Lawley, T.J. and Plotz, P.H.: An immunospecific clearance defect in patients with systemic lupus erythematosus (SLE) related to the levels of circulating immune complexes (Ic). Clinical Research 25:357a, 1977 (abstract).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)

U. S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE
PUBLIC HEALTH SERVICE
**NOTICE OF INTRAMURAL RESEARCH PROJECT**

PROJECT NUMBER

Z01 AM 41031-01 ARB

PERIOD COVERED

July 1, 1976 to September 30, 1977

TITLE OF PROJECT (80 characters or less)

Complement dependent cytotoxic auto-antibodies to neutrophils in patients with Felty's syndrome and Systemic Lupus Erythematosus (SLE).

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

| | | | |
|---|---|---|---|
| PI: | J.A. van Boxel | Visiting Scientist | ARB NIAMDD |
| OTHER: | D. Torretti | Clinical Associate | ARB NIAMDD |
| | R. Tropani | Microbiological Associates | Rockville, Maryland |

COOPERATING UNITS (if any)

Contract with Microbiological Associates. Dr. R.J. Tropani, Head, Special Projects Laboratory

LAB/BRANCH

Arthritis and Rheumatism Branch

SECTION

Connective Tissue Section

INSTITUTE AND LOCATION

NIAMDD, NIH, Bethesda, Maryland 20014

| TOTAL MANYEARS: | PROFESSIONAL: | OTHER: |
|---|---|---|
| ½ | ½ | 0 |

CHECK APPROPRIATE BOX(ES)

[ ] (a) HUMAN SUBJECTS [ ] (b) HUMAN TISSUES (c) NEITHER

[ ] (a1) MINORS [ ] (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The present work is the first to report evidence for complement dependent cytotoxic auto-antibodies to neutrophils in patients with Rheumatoid Arthritis and neutropenia (Felty's Syndrome), and the neutropenia associated with SLE. Using a dye-exclusion microcytotoxicity assay activity was found in eight Felty's syndrome patients, but not in ten patients with rheumatoid arthritis without neutropenia; no significant activity was found in normal controls and subjects with multiple pregnancies or transfusions. Activity could be eluted from column adherent, serum exposed neutrophils by affinity chromatography; eluted protein contained IgG. Indirect immunofluorescent studies showed binding of IgG to neutrophils on exposure to Felty's serum but not normal serum. Antibodies reacted best at 37° unlike antilymphocyte antibodies which are maximally reactive at 4°C. This finding raises the possibility that these antibodies may have a pathogenetic role.



FSS-6040
(Rev. 10-76)

Project Description:

Objectives:

The goal of the present study is to explore the pathogenetic mechanism responsible for the neutropenia of Felty's syndrome, a complication of rheumatoid arthritis. Preliminary studies suggested the presence of antibodies to neutrophils, and the present work was limited to confirming their presence and establishing specificity, Ig class, etc.

Methods Employed:

Two main approaches were used: 1) a dye-exclusion microcytotoxicity assay to demonstrate complement-dependent killing and 2)indirect immunofluorescence to demonstrate specific binding of Ig and the class of Ig bound. Specificity was further demonstrated by affinity chromatography using a cellular immunoabsorbent column. Neutrophils were allowed to adhere to cotton columns, Felty's serum passaged through the columns, the protein eluted at acid pH and then tested for cytotoxicity and immunoglobulin class.

Major Findings:

Major findings were 1) Complement dependent cytotoxic activity to autologous and homologous neutrophils in the serum of eight patients with Felty's syndrome and six with SLE. No activity in ten patients with uncomplicated rheumatoid arthritis, and no significant activity in twelve subjects with multiple pregnancies or multiple transfusions, or twenty normal controls. 2) Binding of IgG to autologous neutrophils following exposure to Felty's serum, but not to normal serum. 3) Elution of IgG and complement dependent cytotoxic activity from neutrophil affinity columns after exposure to serum from Felty's patients. 4) Whereas anti-lymphocytic activity, which was frequently found in these sera as well, was maximal at 4°C and is of the IgM class, anti-neutrophil activity was maximal at 37° and mainly of the IgG class.

Significance to Bio-Medical Research and the Program of the Institute:

Felty's syndrome affects about 1% of patients with rheumatoid arthritis and its pathogenesis is poorly understood. The finding of complement dependent auto-antibodies to neutrophils suggests that these may play a role in the neutropenia of this disorder.

Proposed Course:

Due to the leaving of the PI and Clinical Associate this study will be terminated.

Publications:

Manuscript in preparation.

## METABOLIC DISEASES BRANCH

The general goals of the Branch are to investigate the structure, secretion and biochemical mechanism of action of hormones governing ion transport and mineral metabolism and to apply the knowledge gained to understanding and treating related clinical disorders. Current investigations include hormone-receptor interaction, mechanism of receptor control of adenylate cyclase, cyclic AMP control of ion transport, and regulation of parathyroid hormone secretion. Systems under study include β-adrenergic receptors in several tissues and isolated parathyroid cells. Clinical studies on hyperparathyroidism have utilized extensively research findings from the laboratory. Radioimmunoassays for parathyroid hormone have been applied to determine the hormone in samples obtained selectively by venous catheterization, thereby allowing localization of parathyroid adenomas. Further studies continue on utilizing urinary cyclic AMP clearance as a clinical parameter of parathyroid hormone action. Another study takes advantage of an autologous parathyroid transplant program developed in collaboration with the Surgery Branch, NCI. Autologous transplants to the arm allow direct sampling of draining veins for parathyroid hormone secretion in man under conditions wherein the tissue can be selectively challenged with potential secretagogues or inhibitors of parathyroid secretion.

### β-adrenergic Receptor of the Avian Erythrocytes.

Interactions of catecholamines with β-adrenergic receptors and activation of adenylate cyclase are under study with the plasma membranes of turkey erythrocytes and other cell systems. Specific receptors have now been identified on turkey erythrocytes, parathyroid cells, pineal cells, rat, guinea pig and monkey lung membrane preparations and rat liver membranes.

β-adrenergic receptor sites are identified using a radioiodinated analog of a β-blocker designed for this purpose. Hydroxybenzylpindolol (1-(1-para-hydroxyphenyl-2-methyl-2-propylamino)-3-(4-indolyloxy)-2-propanol) is a high-affinity β-blocker containing a phenolic group. The latter compound can be radioiodinated to high specific activity and binds specifically to receptors. A series of β-adrenergic agonists and inhibitors compete for binding with affinities paralleling biological effectiveness as activators or inhibitors of catecholamine-stimulated adenylate cyclase. Receptor occupancy for agonists correlate linearly with function (cyclic AMP production). The stereospecificity of interaction with the iodinated β-blocking agent and the correspondence between affinity for site and biological effectiveness indicate that binding of iodohydroxybenzylpindolol validly reflects interaction with the β-adrenergic receptor.

Calcitonin receptors were identified on cultured human lymphocytes. The characteristics of these receptors are similar to those on rat renal and calvarial membranes as determined by competitive binding studies. Lymphocyte preparations should prove useful in that they represent a readily obtainable human tissue containing calcitonin receptors. Results with this human material indicated that, although calcitonin polypeptides have undergone

remarkable evolutionary changes in structure and effectiveness, there has been no significant corresponding change in biological nature of calcitonin receptors.
[Drs. Marx and Aurbach, NIAMDD]

Purine nucleotides enhance catecholamine-stimulated adenylate cyclase activity in the erythrocyte membrane. A guanine nucleotide site separate from the catalytic site for ATP regulates adenylate cyclase activity. Although guanine nucleotides (the most effective is guanylylimidodiphosphate, Gpp(NH)p) enhance isoproterenol-activated adenylate cyclase with apparent increase in sensitivity to the agonist, there is nevertheless no change in affinity of the receptor.

Prior incubation of adenylate cyclase with Gpp(NH)p leads to a highly and persistently activated "holocatalytic" state. This activated state, however, shows no change in affinity of ligands for receptor. It is concluded that guanine nucleotides act on adenylate cyclase in the turkey erythrocyte system at a site distal to the receptor. Recent studies indicate that the enzyme reaction itself may involve vicinal dithiol mechanism. Arsenite was found to be an effective inhibitor of the adenylate cyclase reaction and this inhibition was specifically potentiated by dimercaptopropanol (BAL). The mechanism of guanine nucleotide activation of adenylate cyclase was shown not to involve pyrophosphorylation of the enzyme.
[Drs. Spiegel, Downs, Brown and Aurbach, NIAMDD]

Clinical studies are continuing on primary hyperparathyroidism and its familial variants. Detailed family screening and case finding has produced 20 kindreds for analysis. These studies have allowed segregation of the familial variants into two apparently distinct disease syndromes -familial multiple endocrine adenomatosis (MEN I) and familial hypocalciuric hypercalcemia (FHH). FHH was distinguished from MEN I by 1) a higher incidence of hypercalcemia below the age of 20 and virtually a 100% penetrance for hypercalcemia after age 20, 2) milder clinical manifestations - none had recurrent nephrolithiasis or recurrent peptic ulceration, 3) no hypercalciuria, 4) normal basal concentrations of glucagon and gastrin, and 5) poor response to subtotal parathyroidectomy. Distinction between the two syndromes, both inherited as autosomal dominant traits, is important because in FHH the clinical course is generally milder and subtotal parathyroidectomy is less likely to be beneficial. In FHH the ionized and ultrafiltrable calcium concentration in serum is elevated in proportion to the increase in total calcium. These patients have a high filtrable load of calcium associated with a marked decrease in renal calcium clearance.
[Drs. Marx, Spiegel, Brown and Aurbach]

Studies on modes of localizing parathyroid tumors continue. A high degree of success has been obtained in localizing tumors through vascular catheterization procedures. Parathyroid arteriography developed and performed by Dr. John Doppman afforded, in approximately 45% of cases in which it was used, the identification of abnormal masses of tissue subsequently identified as parathyroid. Confirmation of the mass thus identified as parathyroid tissue can be accomplished by verifying high concentrations

of parathyroid hormone by radioimmunoassay in veins draining the lesion. Draining vessels contain concentrations of hormone 2- to 20-fold or more greater than the concentration of parathyroid hormone detected by immunoassay in the peripheral plasma. In cases with single adenomas, this concentration ratio is greatest selectively in the one vein draining the lesion. Contralateral veins show concentrations equal to samples obtained from the general circulation. Patients with hyperplasia of all four glands show concentrations of hormone in the thyroid veins on either side greater than concentrations in peripheral plasma. This technique utilizing specific radioimmunoassays and selective catheterization should be applicable also to determining the source of hormone in ectopic hyperparathyroidism produced by non-parathyroid cancers. Localization of anterior mediastinal parathyroid adenomas has become possible using computed tomography. Three mediastinal adenomas have been treated nonsurgically by percutaneous catheter infusion of agents into the arterial blood supply with two complete and one partial remissions. [Drs. Marx, Spiegel, Brown, and Aurbach, NIAMDD; Dr. Doppman, Radiology Dept., C.C.]

Measurement of intraoperative urinary cAMP excretion in patients undergoing neck exploration for primary hyperparathyroidism has shown that successful removal of abnormal parathyroid tissue is followed by a rapid decline to low levels (<1.5 μmol/g creatinine). In patients in whom surgery was not curative, levels remained elevated. These results suggest that monitoring UcAMP excretion intraoperatively (utilizing techniques for rapid radioimmunoassay of cAMP) could aid in operative management of patients with primary hyperparathyroidism.
[Drs. Spiegel, Marx, Brown and Aurbach, NIAMDD]

A method has been developed for isolating functionally intact bovine and human parathyroid cells. Normal bovine parathyroid glands and human adenomatous or primarily hyperplastic glands are digested with collagenase and DNase to give a cell preparation viable by both morphologic and functional criteria. The cells show inverse proportionate control of hormone secretion in response to calcium although cells from parathyroid adenomas appear to require a higher than normal calcium concentration for 50% inhibition of PTH release. Both human and bovine parathyroid cells contain a beta-adrenergic receptor which has been directly identified by iodohydroxybenzylpindolol binding and characterized in terms of response of cAMP accumulation and PTH release to beta-adrenergic agonists. Recent studies with other ligands elevating cAMP such as prostaglandins, secretin and dopamine also demonstrate a close relationship between cAMP and PTH release in this system. Studies have recently been completed showing a requirement for a permeant anion to support PTH release, similar to that seen in other secretory systems thought to act through exocytosis.
[Drs. Brown, Gardner, Windeck and Pollard, NIAMDD]

A cooperative study between investigators in NIAMDD and members of the staff of Hadassah Medical School-Hebrew University, Jerusalem, involving approximately 300 ambulatory patients with osteoporosis is in progress in Jerusalem. By a combination of dietary survey, x-ray, bone mineral content, medical history and other methods considerable information on the natural history of osteoporosisis is anticipated; in addition, a double-blind study is being made of the possible value of oral administration of high calcium, high phosphate and fluoride in treatment of osteoporosis. Results of this study, now in its fourth year, probably will not be available for about one more year.
[Dr. P. H. Bennett, Dr. G. D. Whedon]

July 1, 1976 - September 30, 1977

Studies in Bone Metabolism

PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

G. D. Aurbach, M.D., Chief, Metabolic Diseases Branch, NIAMDD
A. M. Spiegel, M.D., Clinical Associate, MDB, NIAMDD
G. D. Whedon, M.D., Director, NIAMDD
S. J. Marx, M.D., Senior Investigator, MDB, NIAMDD
E. M. Brown, M.D., Clinical Associate, MDB, NIAMDD
J. Shapiro, M.D., Chief, Section on Endocrinology, Greater Southeast Community Hospital, Washington, D. C.
W. T. Moore, M.D., Washington Hospital Center, Washington, D. C.

Dr. Mones Berman, Mathematical Research Branch, NIAMDD
Greater Southeast Community Hospital, Washington, D. C.

Metabolic Diseases Branch

NIAMDD, NIH, Bethesda, Md. 20014

.50 .25

The objectives of the project on bone metabolism are to investigate the factors influencing mineral storage and loss in demineralizing bone diseases, to investigate the response of the parathyroid gland to rapid changes in blood calcium, and to study the influence of added calcium and phosphate on resorption of bone mineral. The project involves metabolic balance studies. It also involves estimation of pool sizes, turnover rates, bone deposition rates, and absorption rates of calcium in patients by the oral and intravenous administration of tracer doses of $^{47}Ca$, and estimation of bone density by photon absorptiometry.

During the past year, clinical studies of osteoporosis have been continued, using photon absorption densitometry, with observations of the bone mineral content in various populations and of the possible effect thereon of dietary manipulation.

Objectives: (1) To investigate the factors influencing mineral storage and loss in demineralizing bone diseases. (2) To investigate the response of the parathyroid gland to rapid changes in blood calcium. (3) To study the influence of phosphate on resorption of bone mineral.

Methods Employed: (1) Metabolic balance studies in patients with disorders of calcium metabolism, noting the effects on nitrogen, calcium, phosphate and magnesium balances of various dietary intakes of calcium, phosphate and magnesium. (2) Estimation of pool sizes, turnover rates, bone deposition rates, and absorption rates of calcium in patients by the oral and intravenous administration of tracer doses of $^{47}Ca$. (3) Estimation of bone density in the radius by photon absorptiometry.

Major Findings: Studies have continued in order to evaluate the clinical usefulness of photon absorption method of bone mineral analysis. Using this technique, with an iodine-125 source, values for bone mineral content in a normal population of children and adults were determined. These studies demonstrated a fairly linear increase in bone mineral content from birth into the third decade; thereafter bone mineral decreased in both men and women, but at a faster rate in women than in men.

Measurement of bone mineral content in the radius in black populations from birth through adult life disclosed a greater mineral content in blacks compared with whites at all ages. Based on group data (single or few measurements each on many individuals), black females lose mineral in the radius with aging. These studies confirm the clinical impression that osteoporosis is very uncommon in blacks and suggests that as currently defined osteoporosis should not occur as a primary disease in black males. Bone mineral content was determined in black patients with sickle cell anemia. Sixty-four percent of males and 39 percent of females with sickle cell disease have less bone mineral than normal black children of the same age. The ratio of bone mineral content to bone width followed the normal pattern in these children. Loss of bone mineral content did not correlate with severity of anemia or transfusion requirement.

A cooperative study between investigators in NIAMDD and members of the staff of Hadassah Medical School-Hebrew University, Jerusalem, involving approximately 300 ambulatory patients with osteoporosis is in progress in Jerusalem. By a combination of dietary survey, x-ray, bone mineral content, medical history and other methods considerable information on the natural history of osteoporosisis is anticipated; in addition, a double-blind study is being made of the possible value of oral administration of high calcium, high phosphate and fluoride in treatment of osteoporosis. Results of this study, now in its fourth year, probably will not be available for about one more year.
[Dr. P. H. Bennett and Dr. G. D. Whedon]

Publications:

1. Shapiro, J., Moore, T., Jorgensen, H., Reid, J., Epps, C., and Whedon, G.D.: Osteoporosis: evaluation of diagnosis and therapy. Arch. Intern. Med. 135: 563-567, 1975.

2. Whedon, G.D., Lutwak, L., Reid, J., Rambaut, P., Whittle, M., Smith, M., and Leach, C.: Mineral and nitrogen balance study: results of metabolic observations on skylab II 28-day orbital mission. Acta Astronautica 2: 297-309, 1975.

3. Whedon, G.D., Lutwak, L., Rambaut, P., Whittle, M., Leach, C., Reid, J., and Smith, M.: Effect of weightlessness on mineral metabolism: metabolic studies on skylab orbital space flights. Calc. Tissue Res. 21 Suppl: 423-430, 1976.

4. Whedon, G. D., Lutwak, L., Rambaut, P.C., Whittle, M.W., Reid, J., Smith, M.D., Leach, C., Stadler, C.R., and Sanford, D.D.: Mineral and nitrogen balance study observations: the second manned skylab mission. Aviation, Space, and Environmental Medicine 47: 391-396, 1976.

| SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space) | U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT** | PROJECT NUMBER Z01 AM 43,002-12 MD |
|---|---|---|

PERIOD COVERED
July 1, 1976 - September 30, 1977

TITLE OF PROJECT (80 characters or less)
Structure, Secretion and Mechanism of Action of Parathyroid Hormone

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

G. D. Aurbach, M.D., Chief, MDB, NIAMDD
S. J. Marx, M.D., Senior Investigator, MDB, NIAMDD
A. M. Spiegel, M.D., Clinical Associate, MDB, NIAMDD
E. M. Brown, M.D., " "
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H. Pollard, M.D., Reproductive Research Branch, NICHD
J. O. Koehler, M.D., Clinical Associate, MDB, NIAMDD
J. T. Potts, Jr., M.D., Massachusetts General Hospital, Boston
H. T. Keutmann, M.D., "
M. Rosenblatt, M.D., "
R. Windeck, M.D., Visiting Scientist, MDB, NIAMDD

COOPERATING UNITS (if any)
Endocrine Unit, Massachusetts General Hospital

LAB/BRANCH
Metabolic Diseases Branch

SECTION

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Md. 20014

| TOTAL MANYEARS: | PROFESSIONAL: | OTHER: |
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☐ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Little is known about the nature of circulating parathyroid hormone in clinical disease. It is the purpose of this project to isolate parathyroid hormone, determine the structure of the polypeptide, elucidate the mechanism of action of the hormone, and to develop a clinically useful test for circulating parathyroid hormone. From these studies it is expected that one can understand the pathophysiology of certain metabolic diseases of bone and endocrine disturbances. The entire structures of bovine and porcine parathyroid hormone have been determined as well as the first 37 residues of human parathyroid hormone. Synthetic polypeptides representing the first 34 residues of bovine and human parathyroid hormone have been prepared. These molecules show all the biological properties of the native hormonal polypeptide. A highly sensitive radioimmunoassay for the hormone has been developed and is being modified further for potential use as a specific clinical tool. Recent studies show that the mechanism of action of purified hormone is mediated through direct hormonal activation of adenylate cyclase in bone and kidney. An isolated parathyroid cell system has been developed that allows studies of secretagogue for parathyroid hormone.

Objectives: To prepare purified parathyroid hormone in pilot-plant quantities; to study chemical properties and structure of the hormone; to relate chemical properties and structure to biological activity and to study the mechanism of action of the hormone; to develop a clinically useful test for circulating parathyroid hormone; to study the physiological regulation of secretion of the hormone.

Methods Employed: Pure parathyroid hormone is prepared from phenol extracts by methods previously described from this laboratory. The hormone is assayed in vivo in rats, in vitro by measuring hormonal activation of adenylate cyclase, or by a highly sensitive radioimmunoassay. Adenylate cyclase is measured by determining conversion of $\alpha$-$^{32}$P-labeled ATP to cyclic 3',5'-AMP. Endogenous concentration of cyclic 3',5'-AMP or cyclic 3',5'-GMP is determined by radioimmunoassay.

Major findings: mechanism of action. Our earlier studies showed that the parathyroid status of animals is an important factor regulating excretion of cyclic AMP in the urine. This finding led to development of the thesis that cyclic AMP is involved in the mediation of parathyroid hormone action. Subsequent work showed that parathyroid hormone in vitro activates adenylate cyclase in the kidney as well as in bone, the two organs known to be physiological receptors for action of the hormone. Adenylate cyclase in the kidney is found in two different anatomic zones; adenylate cyclase in the cortex responds specifically to parathyroid hormone whereas that in the medulla responds to vasopressin. This work and numerous corroborative studies in other laboratories established that the receptor for parathyroid hormone in kidney resides within the renal cortical tubule as had been suspected but not proven from indirect in vivo physiological experiments.

Human parathyroid hormone. Parathyroid hormone has been purified from human parathyroid adenomas. Earlier studies proved that the human hormone is different immunologically from the bovine hormone. Amino acid analyses of the hormone purified from each source indicated approximately eight amino acid substitutions in the human hormone. The amino acid sequence has been completed for the first 37 amino acids at the amino-terminal end of the molecule (Drs. Potts and Rosenblatt). Based on this analysis, Drs. Potts and Rosenblatt (Boston) synthesized the tetratriacontapeptide representing the amino terminus of the molecule. The product is fully as active in vitro and in vivo as the hormone isolated from human parathyroid adenomas. [G.D.Aurbach; Drs. Potts, Rosenblatt and Keutmann]

Efforts are continuing to collect large amounts of human parathyroid tissue from which further quantities of human hormone can be purified. With the highly sophisticated techniques now available for sequence analysis, it is projected that if a further 1 to 2 mg of human parathyroid hormone can be isolated in pure form it will be possible to determine the amino acid sequence in the C-terminal region represented by the remainder of the molecule, residues 38-84.

Parathyroid cells in vitro. Methods have been developed for isolating functionally intact bovine parathyroid cells. Finely minced tissue is digested for 30-60 minutes with collagenase and DNase and periodically disrupted by vigorous pipetting. The resulting cell population is indistinguishable from intact bovine tissue by electron microscopy and shows a similar inversely proportionate relationship of parathyroid hormone (PTH) release to calcium. A similar method has been devised to prepare viable dispersed cells from human parathyroid adenomas and primary hyperplasia.

Isolated bovine parathyroid cells also have an intact $\beta_2$-adrenergic receptor which has been characterized by binding studies with the iodinated β-adrenergic blocker $^{125}$I-hydroxybenzylpindolol ($^{125}$I-HYP). Bovine parathyroid cells contain 5000-10,000 β-adrenergic receptors/cell and there is a close correlation between effects of agonists and antagonists on binding of $^{125}$I-HYP, accumulation of cAMP, and release of PTH. Several other secretagogues have now been defined which appear to act through alterations in intracellular cAMP. Dopamine stimulates cellular cAMP 20-30 fold through a receptor which is clearly distinguishable from the β-adrenergic receptor by studies with specific antagonists. Like β-adrenergic agonists, dopamine stimulates PTH release 2-4 fold at early times (5-30 minutes). Prostaglandins of the E-series, and the gastrointestinal hormone secretin also stimulate cAMP accumulation and PTH release. The close relationship between intracellular cAMP and PTH release is readily shown by correlating intracellular cAMP and the corresponding PTH release in response to a variety of agents altering cAMP (lowering calcium concentration, phosphodiesterase inhibitors, isoproterenol, and dopamine).

Recent collaborative studies (Dr. Pollard, NIAMDD) have demonstrated a requirement for a permeant anion ($Cl^-$ or $OH^-$) to support PTH release from dispersed parathyroid cells. The anion-stimulated release is competitively inhibited by classical anion transport blockers such as probenecid, suggesting that anion transport may regulate exocytosis in this system. These findings are analogous to those in other exocytotic systems analyzed to date (isolated chromaffin granules, platelet serotonin release).

Studies employing isolated cells from pathologic human parathyroid glands removed at the time of surgery for hyperparathyroidism suggest a variety of abnormalities in function. The majority of cell preparations from patients with both adenomas and primary hyperplasia suppress to 20-40% of their maximal hormone output, but adenomas appear to require a higher than normal calcium to suppress. The cells from several patients show no response to isoproterenol with PTH release although they do respond with about 10% of the cAMP response of normal bovine tissue.

These studies indicate the utility of isolated parathyroid cells for investigating normal or abnormal parathyroid physiology. The mechanism of secretagogue-stimulated PTH release may be investigated in terms of hormone binding, intracellular mediators, and the synthesis and release of PTH.
[Drs. Brown, Gardner, Windeck and Pollard]

Significance to Biomedical Research and Program of the Institute: Same as for July 1968-June 1976.

Proposed Course of Project: Same as for July 1968-June 1976.

Publications:

1. Aurbach, G.D.: Hormone receptors in the kidney. In Bolis, L., Hoffman, J., and Leaf, A. (Eds.): Membranes and Diseases. New York, Raven Press, 1976, pp. 291-299.

2. Desbuquois, B., and Aurbach, G.D.: Use of polyethylene glycol to separate free and antibody-bound peptide hormones in radioimmunoassays. In Antoniades, H.H. (Ed.): Hormone in Human Plasma. Cambridge, Massachusetts, Harvard University Press, 1976, pp. 155-159.

3. Desbuquois, B., and Aurbach, G.D.: Radioimmunoassay of insulin using polyethylene glycol. In Antoniades, H.M. (Ed.): Hormones in Human Blood. Boston, Harvard University Press, 1976, pp. 200-203.

4. Brown, E.M., Hurwitz, S., and Aurbach, G.D.: Preparation of viable isolated bovine parathyroid cells. Endocrinology 99: 1582-1588, 1976.

5. Brown, E.M., Hurwitz, S., and Aurbach, G.D.: Beta-adrenergic stimulation of cyclic AMP content of parathyroid hormone release from isolated bovine parathyroid cells. Endocrinology 100: 1696-1702, 1977.

6. Brown, E.M., Hurwitz, S., Woodard, C.J., and Aurbach, G.D. Direct identification of beta-adrenergic receptors on isolated bovine parathyroid cells. Endocrinology 100: 1703-1709, 1977.

7. Aurbach, G.D., Brown, E.M., and Marx, S.J.: Receptors and cyclic nucleotides in secretion and action of parathyroid hormone. In Robertson, W.G. (Ed.): Proceedings of XII European Symposium on Calcified Tissues, York, England, July 1976, in press.

8. Aurbach, G.D., and Phang, J.M.: Vitamin D, parathyroid hormone and calcitonin. In Mountcastle, V.B. (Ed.): Medical Physiology, 14th Edition, Vol. III. Saint Louis, The C. V. Mosby Co., in press.

| SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space) | U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT | PROJECT NUMBER Z01 AM 43,003-12 MD |
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PERIOD COVERED
July 1, 1976 - September 30, 1977

TITLE OF PROJECT (80 characters or less)

Studies on the Mode of Action of Thyrocalcitonin

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

G. D. Aurbach, M.D., Chief, Metabolic Diseases Branch, NIAMDD
Stephen J. Marx, M.D., Senior Investigator, MDB, NIAMDD
Edward M. Brown, M.D., Clinical Associate, MDB, NIAMDD

COOPERATING UNITS (if any)

None

LAB/BRANCH
Metabolic Diseases Branch

SECTION

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Md. 20014

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The purpose is to study the interaction of calcitonin with its specific receptor target organs. The current investigations should provide further insight into the structure function relationship in calcitonin. Calcitonin is a small polypeptide hormone and therefore lends itself well to studies using synthetic peptide fragments. The system is also useful for characterizing hormone receptors in kidney, bone and other tissues. Studies are in progress to characterize further the interaction of calcitonin with tissue receptors. It also will be of interest to solubilize the receptors and characterize them chemically.

Objectives: To study the interaction of calcitonin with its specific receptor target organs.

Methods Employed: The interaction of calcitonin with its receptors has been evaluated by determining adenylate cyclase as well as binding of radioiodinated calcitonin to membranes isolated from renal tissue and to lymphoid cells isolated from human and other species or grown in tissue culture.

Major Findings: A purification procedure for kidney membranes has been developed. It has been shown that the calcitonin receptor from the kidney is concentrated at the corticomedullary junction. The membranes which bind calcitonin and contain calcitonin-sensitive adenylate cyclase show low specific activities for renal brush border enzymes, indicating that calcitonin receptors are localized at an anti-luminal aspect of renal cells or at a site that does not contain brush borders. Utilizing a series of calcitonin analogs, dose response curves were compared for binding to receptors from kidney and bone. The dose response curves in the two tissues are virtually superimposable, suggesting that the calcitonin receptor in the two organs has a similar molecular structure. The interaction of the hormone-receptor complex with the adenylate cyclase enzyme is modified by the presence of guanine nucleotides. In the presence of GMPPNP the threshold for hormonal activation is lowered.

The relative renal responses to calcitonin analogs are similar whether determined by competitive binding or adenylate cyclase activation. Thus potency differences of the analogs tested are largely dependent upon effectiveness at the receptor level. Activity of the adenylate cyclase enzyme of human lymphocytes was not modulated by ambient or bound calcitonin.

Calcitonin receptors were identified on cultured human lymphocytes. The characteristics of these receptors are similar to those on rat renal and calvarial membranes as determined by competitive binding studies. The lymphocyte receptors, however, do not appear capable of modulating adenylate cyclase enzyme in that cyclic AMP was not detected after adding calcitonin to intact cells. Lymphocyte preparations should prove useful, however, in that they represent a readily obtainable human tissue containing calcitonin receptors. Results with this material indicated that, although calcitonin polypeptides have undergone remarkable evolutionary changes in structure and effectiveness, there has been no significant corresponding change in biological nature of calcitonin receptors.

Significance to Biomedical Research and Program of the Institute: The current investigations should provide further insight into the structure-function relationship in calcitonin. Calcitonin is a small polypeptide hormone and therefore lends itself well to studies using synthetic peptide fragments. The system is also useful characterizing hormone receptors present in kidney, bone, and other tissues.

Proposed Course of Project: Further studies are in progress to characterize further the interaction of calcitonin with its tissue receptors. It also

will be of interest to solubilize calcitonin receptors and further characterize them. Purified lymphoid cell types are being characterized with respect to the density of calcitonin receptors on their surface. The whole cell system also may be applied to evaluate the regulation of receptor metabolism *in vitro* and in human disease states.

Publications:

None

| SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space) | U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT** | PROJECT NUMBER Z01 AM 43,004-12 MD |
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PERIOD COVERED
July 1, 1976 - September 30, 1977

TITLE OF PROJECT (80 characters or less)

Studies on pseudohypoparathyroidism and related disorders

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

G. D. Aurbach, M.D., Chief, Metabolic Diseases Branch, NIAMDD
S. J. Marx, M.D., Senior Investigator, MDB, NIAMDD
A. M. Spiegel, M.D., Clinical Associate, MDB, NIAMDD
E. M. Brown, M.D., " "
J. O. Koehler, M.D. " "

COOPERATING UNITS (if any)

LAB/BRANCH
Metabolic Diseases Branch

SECTION

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Md. 20014

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

In 1942 Albright and his associates described the features of a new clinical syndrome "pseudohypoparathyroidism." Patients with this disorder differ from those with idiopathic hypoparathyroidism in that they have characteristic constitutional features and they do not respond to exogenous parathyroid extract. Our findings that parathyroid hormone action is likely mediated through activation of adenylate cyclase, led us to test cases of pseudohypoparathyroidism by giving intravenous parathyroid hormone and measuring cyclic 3',5'-AMP in the urine. In normal subjects as well as usual forms of hypoparathyroidism, parathyroid hormone causes a 10- to 60-fold increase in urinary cyclic AMP (cAMP). This response is abnormal (slight or no increased cAMP excretion) in pseudohypoparathyroidism, indicating that there is a defective parathyroid hormone-adenylate cyclase complex in the kidney in this disorder.



PHS-6040

Project Description. In 1942 Albright and his associates described the features of a new clinical syndrome "pseudohypoparathyroidism." Patients with this disorder differ from those with idiopathic hypoparathyroidism in that they have characteristic constitutional features and they do not respond to exogenous parathyroid extract. Albright proposed that this disorder was attributable to lack of end organ sensitivity to normally-secreted endogenous parathyroid hormone. Our findings that parathyroid hormone action is likely mediated through activation of adenylate cyclase, led us to test cases of pseudohypoparathyroidism by giving intravenous parathyroid hormone and measuring cyclic 3',5'-AMP in the urine. Pseudopseudohypoparathyroidism is a variant of the disorder and may occur in the same families with pseudohypoparathyroidism. Pseudopseudohypoparathyroidism is characterized by similar constitutional features but no clinical evidence of hypoparathyroidism. Several other syndromes including Gardner's syndrome, basal cell nevus syndrome, syndrome of calcification of basal ganglia, and vitamin D-resistant osteomalacia have also been reported in the past as resistant to the phosphaturic action of parathyroid hormone. Current studies have been extended to include testing patients in these categories as well.

Methods: Parathyroid hormone purified in our laboratories was sterile-filtered for clinical use through courtesy of the N.I.H. Pharmacy. Radioimmunoassay of parathyroid hormone was carried out as described previously. Measurement of 3',5'-AMP was performed according to the procedure devised in this laboratory.

Major Findings: In normal subjects, parathyroid hormone causes a 10- to 60-fold rise in urinary excretion of cyclic AMP within 30 minutes or less after intravenous administration of the hormone. A similar response has been observed in idiopathic, hypoparathyroid, surgical hypoparathyroid, and pseudopseudohypoparathyroid subjects (the latter category show the constitutional changes but do not have laboratory evidence of hypoparathyroidism). Parathyroid hormone caused little or no increase in excretion of cyclic AMP in patients with pseudohypoparathyroidism. In one case, studied through the cooperation of a clinical investigator at another hospital, there was a moderate rise in excretion of cyclic AMP; however, the diagnosis in this instance may be incorrect, and as yet we have not had the opportunity of seeing the patient at the Clinical Center. Patients with this disorder who fail to respond to parathyroid hormone showed a similarly defective response after prolonged calcium infusion. Parathyroid hormone was detectable in the plasma of pseudohypoparathyroid subjects before infusion of calcium but not after. This experiment tended to rule out the possibility that an abnormal endogenously secreted hormone immunologically reactive but biologically inert interfered with the action of exogenous parathyroid hormone.

The best evidence to date is that pseudohypoparathyroidism represents a genetic defect inherited as a sex-linked, dominant trait. There have been no documented cases of direct male-to-male transmission of the disease and the sex incidence is approximately 2:1 female-to-male. Several pedigrees

have been described wherein pseudophyoparathyroid subjects were progeny of pseudopseudohypoparathyroid parents or vice versa. One family studied at the N.I.H. included a mother with pseudopseudohypoparathyroidism and her daughter with pseudohypoparathyroidism. The daughter failed to respond to exogenous hormone but the mother responded normally. We have also observed that, although the response to pseudopseudohypoparathyroidism is normal to exogenous hormone, there is a significantly increased rate of baseline excretion of cyclic AMP in this group. This observation may be of importance in further delineation of the precise genetic mechanism involved in transmission of the disorder. It appears likely that the cause of the disorder may be the existence of genetically deficient or defective adenylate cyclase in the bone and kidney of these subjects.

There is an association of hypothyroidism with pseudohypoparathyroidism and in a few such cases the hypothyroidism has been attributed to selective deficiency of thyrotropin. It seems possible that hypothyroidism in pseudohypoparathyroidism might reflect an abnormal receptor adenylate cyclase complex in the central nervous system or anterior pituitary analogous to the defect in the receptor for parathyroid hormone in kidney and bone. Three patients with coexistent hypothyroidism and pseudohypoparathyroidism were given thyrotropin-releasing hormone as part of a study designed to localize the site of the thyroid defect. One case showed an abnormally low basal concentration of thyrotropin in plasma, but gave a normal response to thyrotropin-releasing hormone. In the two other cases, a mother and daughter, there were high basal concentrations of thyrotropin in plasma. The mother was tested with TRH and showed the exaggerated response to TSH secretion characteristic of primary hypothyroidism. The defect in the first case appears to be at the level of the hypothalamus or higher. The defect in the second two cases may represent an abnormality of the thyroid receptor for TSH itself.

Patients with Cushing's syndrome, basal cell nevous syndrome, syndrome of calcification of the basal ganglia, and vitamin D-resistant osteomalacia showed normal responses to parathyroid hormone as determined by urinary excretion of 3',5'-AMP. It was concluded that the classical phosphaturic response to parathyroid hormone is neither precise nor sensitive enough to discriminate normal subjects from those refractory to parathyroid hormone.

Kidney tissue was obtained from a patient with pseudohypoparathyroidism who died suddenly with complications of a massive pulmonary embolus. It was demonstrated *in vitro* that the membrane fraction of this kidney contained adenylate cyclase activity which was stimulatable by parathyroid hormone. Thus, it was concluded that pseudohypoparathyroidism did not represent a total lack of the renal parathyroid hormone-sensitive adenylate cyclase system.

Significance to Biomedical Research and Program of the Institute: Same as reported for July 1, 1968-June 30, 1976.

Proposed Course of Project: Same as reported for July 1, 1968-June 30, 1976.

Publications:

1. Spiegel, A.M., Di Chiro, G., Gorden, P., Ommaya, A.K., Kolins, J., and Pomeroy, T.C.: Diagnosis of radiosensitive hypothalamic tumors without craniotomy. Ann. Intern. Med. 85: 290-293, 1976.

2. Marx, S.J., and Aurbach, G.D.: Editorial. N. Engl. J. Med. 296: 169, 1977.

| SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space) | U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT** | PROJECT NUMBER Z01 AM 43,005-12 MD |
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PERIOD COVERED
July 1, 1976 - September 30, 1977

TITLE OF PROJECT (80 characters or less)
Hormone Receptors, Adenylate Cyclase and Cyclic AMP-Mediated Ion Transport

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

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E. M. Brown, M.D., " "
R. W. Downs, Jr., M.D. " "
J. D. Gardner, M.D., Chief, Section on Gastroenterology, DDB, NIAMDD

COOPERATING UNITS (if any)
Section on Gastroenterology, Digestive Diseases Branch, NIAMDD

LAB/BRANCH
Metabolic Diseases Branch

SECTION

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

To isolate and purify specific hormone receptors in particular receptor tissues; to map function of the receptor against activation of adenylate cyclase; to determine the nature of the link between generation of cyclic 3',5'-AMP and solute transport in particular cell systems. β-adrenergic receptors have been identified using radioiodinated hydroxybenzylpindolol (1-(1-para-hydroxyphenyl-2-methyl-propylamino)-3-(4-indolyloxy)-2-propanol), a high-affinity β-blocker. This compound binds specifically to β-receptor sites on cell membranes. Kinetic and equilibrium studies identified a single class of high affinity ($2\text{-}4 \times 10^{10}\ M^{-1}$) receptor sites on turkey erythrocyte membranes, 200-400 sites per cell. A series of β-adrenergic agonists and inhibitors compete for this binding site with affinities paralleling biological effectiveness as activators or inhibitors of catecholamine-stimulated adenylate cyclase. The stereospecificity and the correspondence between affinity for site and biological effectiveness indicate that binding of iodohydroxybenzylpindolol validly reflects interaction with β-adrenergic receptors in several cell systems.



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Objectives: To isolate and purify specific hormone receptors in particular receptor tissues; to map function of the receptor against activation of adenylate cyclase; to determine the nature of the link between generation of cyclic 3',5'-AMP and solute transport in particular cell systems.

Methods Employed: Polypeptide and catecholamine hormones are purified and iodinated by standard methods or methods developed in the laboratory specifically for that purpose. Rate of association of hormones is determined by incubating plasma membrane fractions or whole cell with labeled hormones. The kinetics of binding is determined by rate measurements as well as assessment of steady state conditions. Results are analyzed by double reciprocal, Scatchard, or four-parameter logistic (Rodbard) models utilizing computer programs based on these models. Specific hormone binding is defined in terms of dissociation constant ($K_D$) of the system. Physiologically significant binding shows $K_D$'s compatible with the dose-response relationship recognized for the hormone at the physiological level.

The interaction of catecholamines with the β-adrenergic receptor and activation of adenylate cyclase were studied with the plasma membrane of turkey erythrocytes and mammalian reticulocytes. Interaction of β-adrenergic agonists with receptor in turkey erythrocytes causes specific stimulation of sodium transport. This effect is mediated through the adenylate cyclase-cyclic 3',5'-AMP (cAMP) system and can be reproduced by adding exogenous cAMP.

Incubation of turkey erythrocyte membranes with the GTP analog, GMPPNP, and isoproterenol leads to persistent activation of adenylate cyclase ("holocatalytic state") even after repeated sedimentation and resuspension of membranes in buffer without agonist. Conditions that prevent GMPPNP binding (reduced temperature, GTP, 1 mM EDTA) prevent formation of the holocatalytic state. Beta-adrenergic blockers can prevent formation of this state but are ineffective in reversing it once it has been formed.

The affinity and number of β-adrenergic receptors as determined with I-HYP are identical in the holocatalytic state as compared with control membranes. This indicates that formation of the holocatalytic state with GMPPNP involves a modification in the adenylate cyclase complex distal to the hormone receptor.

The mechanism of guanine nucleotide activation of adenylate cyclase has been studied in turkey erythrocyte and rat reticulocyte membranes. When an ATP analog, App(NH)p, is used as the enzyme substrate, stimulation by catecholamines is dependent upon the presence of guanine nucleotides. A novel GTP analog, $Gp(CH_2)pp$, was found capable of persistently activating adenylate cyclase, excluding the possibility that guanine nucleotides act by pyrophosphorylating the enzyme. Results suggest that GTP binds to sites on the membranes and leads to enzyme activation that is terminated by cleavage of GTP. Compounds resistant to cleavage by the membranes, e.g. Gpp(NH)p and $Gp(CH_2)pp$, lead to persistent enzyme activation.

Adenylate cyclase activity is sensitive to monothiol inhibitors such as organic mercurials and N-ethyl maleimide. Vicinal dithiol inhibitors, arsenite and cadmium were found to inhibit adenylate cyclase activity in turkey erythrocyte and rat reticulocyte membranes. Cadmium proved to be a potent inhibitor ($K_i \sim 10^{-4}$ M). Inhibition by cadmium could be prevented by chelators such as EGTA or EDTA. Arsenite alone was a weak inhibitor (7 mM → only 70% inhibition) but its effect was greatly potentiated by equimolar 2,3 dimercaprol (BAL). Monothiols such as mercaptoethanol were much less effective in potentiating arsenite inhibition. Excess BAL could prevent or reverse both arsenite and cadmium inhibition and was more effective than mercaptoethanol. The inhibitors were effective against basal, catecholamine, and fluoride-stimulated membrane-bound enzyme as well as against a detergent solubilized enzyme preparation, but had no significant effect on binding to the β-adrenergic receptor. These results indicate that vicinal dithiol groups are involved in the catalytic function of adenylate cyclase.

β-adrenergic receptor sites have been identified using a radioiodinated analog of a β-blocker designed for this purpose. Hydroxybenzylpindolol (1-(1-para-hydroxyphenyl-2-methyl-2-propylamino)-3-(4-indolyloxy)-2-propanol) is a high affinity β-blocker containing a phenolic group. The latter compound can be radioiodinated to high specific activity and binds specifically to a site on turkey erythrocyte membranes. A series of β-adrenergic agonists and inhibitors compete for this binding site with apparent affinities paralleling biological effectiveness as activators or inhibitors of catecholamine-stimulated adenylate cyclase. The stereospecificity of interaction with the iodinated β-blocking agent and the correspondence between affinity for site and biological effectiveness reflects validly interactions with β-adrenergic receptors.

β-Adrenergic Receptor of Mammalian Erythrocytes.

The mature nonnucleated mammalian erythrocyte lacks adenylate cyclase activity. Immature rat erythrocytes (reticulocytes), however, possess significant adenylate cyclase activity which can be stimulated by catecholamines, GMPPNP and sodium fluoride. The reticulocytes contain β-adrenergic receptors identified with radioiodinated hydroxybenzylpindolol (I-HYP) as ligand. Antagonists and agonists display stereospecificity ((-)>>(+)) and orders of potency as inhibitors of I-HYP binding with rat reticulocyte membranes that parallel effects on adenylate cyclase activity. During red cell maturation, there is a rapid loss (∿ 10% remaining after 1 week) of adenylate cyclase activity (including basal catecholamine- and fluoride-stimulated enzyme). β-adrenergic receptors are lost at a slower rate than adenylate cyclase activity during maturation. These studies provide evidence that hormone receptor and catalytic moiety are discrete entities.

Significance to Biomedical Research and Program of the Institute: There are a number of disease states which probably represent abnormalities of hormone receptors or the coupling between receptor, generation of cyclic AMP and physiological response to the hormone. This project represents the possible development of a model system wherein the connection between receptor binding

of hormone, generation of cyclic AMP and transport can be investigated. Diseases such as nephrogenic diabetes insipidus or pseudohypoparathyroidism can better be explained once the inter-connecting links between these cellular events are understood.

Proposed Course of Project: Continuing studies are directed towards isolation of the receptors for particular hormones in receptor cells; solubilization and purification of adenylate cyclase and of the receptor; analysis of the interconnecting substance between binding and adenylate cyclase activation; isolation of the guanine nucleotide binding protein; identification of the system activated by cyclic AMP in stimulating transport of solute.

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| SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space) | U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT | PROJECT NUMBER Z01 AM 43,006-02 MI |
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PERIOD COVERED
July 1, 1976 - September 30, 1977

TITLE OF PROJECT (80 characters or less)
Study of Hyperparathyroidism: Etiology, diagnosis and treatment.

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

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LAB/BRANCH
Metabolic Diseases Branch

SECTION

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Md. 20014

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CHECK APPROPRIATE BOX(ES)

☒ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The project goal is the evaluation and treatment of hyperparathyroidism. Patients with persistent postoperative hyperparathyroidism are referred for evaluation and treatment. Hereditary hyperparathyroidism in particular is under investigation in the hopes of delineating hereditary molecular abnormalities in glandular regulation, as exemplified in the multiple endocrine neoplasia syndromes. Evaluation ranges from epidemiologic studies of families to in-house studies of patients and to in vitro analyses of excised tissue. Techniques currently being employed and improved include radioimmunoassay of parathyroid hormone, selective arteriography and selective venous sampling for parathyroid hormone, parathyroid gland cryopreservation and autotransplantation, and transcatheter parathyroid gland infarction.

Objectives: To study the regulation of parathyroid gland function. To develop and evaluate diagnostic tests of parathyroid function. To develop new methods for localization of hyperfunctioning parathyroid tissue. To evaluate methods of treatment of hyperparathyroidism.

Methods employed: Patients with suspected hyperparathyroidism are referred for evaluation and treatment. Evaluation includes radioimmunoassay of circulating parathyroid hormone, and of plasma and urinary cyclic AMP. Where appropriate, preoperative evaluation includes selective arteriography and venography with selective sampling for PTH determination and computerized tomography of neck and mediastinum. In selected cases adenomatous parathyroid glands are ablated by transarterial infusion of various agents. Parathyroid exploration is done by the Surgery Branch, NCI. If postoperative hypoparathyroidism is considered highly likely, parathyroid tissue may be simultaneously autotransplanted to a forearm muscle. Excised parathyroid tissue either fresh or cryopreserved is evaluated in vitro after preparation of dispersed cells. If postoperative hypoparathyroidism is considered possible, tissue is cryopreserved for subsequent autotransplantation. Techniques for cryopreserving and grafting parathyroid tissue are being studied in an experimental model with adult dogs.

Major findings: Diagnosis and clinical findings: A large number of patients with primary hyperparathyroidism have been treated and their clinical and chemical findings have been described. Weakness was noted more commonly than had previously been appreciated and muscle biopsy showed atrophy of type I and especially type II fibers in a manner diagnostic of denervating disease. Studies of neuromuscular function in subjects with secondary hyperparathyroidism showed similar clinical and histologic findings. The most useful diagnostic tests for hyperparathyroidism have been hypercalcemia associated with nonsuppressed or increased parathyroid hormone concentrations in peripheral blood and increased urinary excretion of cyclic AMP. These findings revert rapidly to normal following successful parathyroidectomy. Intestinal absorption of calcium is increased preoperatively and undoubtedly has a central role in causing hypercalcemia.
[G.D.Aurbach, S.J.Marx, A.M.Spiegel, E.M.Brown, D.G.Gardner, and J.O.Koehler, MDB,NIAMDD: J.Sode, U.S.Naval Hospital; M.Brennan,Surgery Branch, NCI]

Several studies have utilized urinary cyclic AMP excretion as a parameter of parathyroid hormone action:

Measurement of intraoperative urinary cAMP excretion in patients undergoing neck exploration for primary hyperparathyroidism has shown that successful removal of abnormal parathyroid tissue is followed by a rapid decline to low levels (<1.5 μmol/g creatinine). In patients in whom surgery was not curative, levels remained elevated. These results suggest that monitoring UcAMP excretion intraoperatively (utilizing techniques for rapid radioimmunoassay of cAMP) could aid in operative management of patients with primary hyperparathyroidism.

Lithium has several actions (e.g. inhibition of antidiuretic hormone action) that have led to the hypothesis that it is a general inhibitor of adenylate cyclase and may be useful in treating diseases characterized by excessive hormone activation of this enzyme. The effects of lithium on PTH activation of renal adenylate cyclase were studied in patients receiving lithium for treatment of manic depressive disorders and in patients with primary hyperparathyroidism. We found no inhibition by lithium of the rise in UcAMP excretion following PTH infusion and no effect of lithium on serum calcium or tubular reabsorption of phosphate in patients with primary hyperparathyroidism. Lithium appears to be unable to inhibit PTH activated adenylate cyclase at doses that can be achieved clinically.

Familial hyperparathyroidism: Surveys of families of patients with primary parathyroid hyperplasia show that hereditary disease is very common (approximately 50% in our series of 25 index cases with primary parathyroid hyperplasia). In two large families a novel form of hyperparathyroidism was found wherein hypercalcemia occurs without hypercalciuria. Unlike typical cases of familial hyperparathyroidism, the penetrance of hypercalcemia approached 100% in the first two decades with severe expression even in one neonate. Review of results of surgery at the N.I.H. and elsewhere has shown that all of 6 patients in this category treated by subtotal parathyroidectomy had persistent postoperative hypercalcemia. While elevated concentrations of glucagon and/or gastrin were common in subjects with familial multiple endocrine neoplasia type I, these were not elevated in hypercalcemic members of these two families.
[S.J.Marx, A.M.Spiegel, E.M.Brown, D.G.Gardner, J.O.Koehler, and G.D.Aurbach, MDB,NIAMDD; J.Gardner, DDB,NIAMDD; L.Recant, V.A.Hospital,Washington,D.C.]

Application of new radiologic techniques: A series of studies has been carried out utilizing selective venous catheterization and a radioimmunoassay for parathyroid hormone for localization of parathyroid adenomas. A high degree of success has been obtained in localizing these tumors preoperatively. The catheter is passed through the femoral vein and thence through the major veins to reach the neck where selective samples are taken from each inferior and superior thyroid vein. Radioimmunoassays show that veins draining parathyroid adenomas frequently contain concentrations of hormone 10- to 20-fold or more greater than the concentration in the peripheral plasma. In cases with single adenomas, this concentration ratio is greatest selectively in one vein draining the adenoma. The contralateral vein shows concentrations equal to samples from the general circulation. Patients with hyperplasia of all four glands show concentrations of hormone in the thyroid veins on each side greater than concentrations in peripheral plasma. Usually in these cases the concentration ratio is not as high as for single adenomas. This technique utilizing specific radioimmunoassays and selective catheterization should be applicable also to determining the source of hormone in ectopic hyperparathyroidism produced by nonparathyroid cancers.

Thyroid and internal mammary arteriography enables preoperative identification of hyperfunctioning parathyroid tissue in 30% or more of cases and is also helpful in defining venous drainage to facilitate selective catheterization.

In three cases of mediastinal parathyroid adenoma treatment was accomplished by transarterial infusion of hypertonic glucose, gelfoam or autologous clot to occlude the catheterized parathyroid artery. Two appear fully cured and one partially. There was no significant morbidity to these patients who would otherwise have required sternotomy.
[G.D.Aurbach, S.J.Marx, A.M.Spiegel, E.M.Brown, D.G.Gardner, and J.O.Koehler, MDB,NIAMDD: J.Doppman, Radiology Dept., C.C., NIH]

Autotransplantation and cryopreservation of parathyroid: Persistent postoperative hyperparathyroidism or hypoparathyroidism are potential problems in patients with primary parathyroid hyperplasia or previous unsuccessful parathyroid exploration. These problems may be lessened if parathyroid tissue can be cryopreserved. We have developed experience with cryopreservation and autotransplantation of parathyroid glands in normal dogs and also in a few patients who developed permanent hypoparathyroidism postoperatively. Avoidance of tissue trauma or rapid changes in temperature or pH was essential for tissue preservation. Canine parathyroid tissue has been successfully autotransplanted after cryopreservation for as long as nine months and function has been documented by measuring release of immunoreactive PTH and by induction of hypocalcemia following graft excision.
[G.D.Aurbach, S.J.Marx, A.M.Spiegel, E.M.Brown, D.G.Gardner, and J.O.Koehler, MDB,NIAMDD; G.Leight, H.Sears, and M.Brennan, Surgery Branch, NCI]

Significance to Biomedical Research and Program of the Institute: As an outgrowth of these studies, methods of localizing and treating hypersecreting tissues of several types are being improved. Hyperparathyroidism is a prototype of endocrine hyperfunction. Some patients have hyperparathyroidism in combination with hereditary hyperfunction of other endocrine glands including pituitary and pancreas. These hyperfunctions are usually benign but occasionally become cancerous. By studying families with hereditary disease we hope to gain insight into specific molecular lesions that cause loss of normal cellular regulation in these tissues. This collaborative program offers comprehensive treatment for a select group of patients who have proven difficult or impossible to treat in other centers in the United States and abroad.

Proposed Course of Project: Patients with suspected primary hyperparathyroidism will be accepted for evaluation and treatment. We will continue to study familial patterns of hyperparathyroidism and efforts will continue towards improving techniques for assessment of parathyroid hormone by radioimmunoassay or bioassay. We will continue to explore new methods of localizing hyperfunctioning parathyroid tissue. The applicability of parathyroid embolization and the role of parathyroid autotransplantation will be investigated further. Patients in the entire program will provide a source of abnormal parathyroid glands to be studied in detail in vitro. Patients with parathyroid transplants will be ideal test subjects for putative parathyroid secretagogues.

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Annual Report of the Section on Gastroenterology, Digestive Diseases Branch
National Institute of Arthritis, Metabolism and Digestive Diseases

During the past year we have continued our studies of the biochemical basis of action of gastrointestinal hormones on pancreatic acinar cells and have initiated a study of the biochemical basis of action of gastric secretagogues.

Dispersed acinar cells prepared from guinea pig pancreas have two functionally distinct classes of plasma membrane receptors for VIP and secretin. One class of receptors has a high affinity for VIP and a low affinity for secretin. Occupation of this receptor causes activation of adenylate cyclase, increased cellular cyclic AMP and stimulation of amylase secretion. The other class of receptors has a low affinity for VIP and a high affinity for secretin. Occupation of this receptor also causes activation of adenylate cyclase and increased cellular cyclic AMP; however, the functional significance of these changes are not known.

In pancreatic acinar cells cholinergic agents and cholecystokinin as well as related peptides cause release of membrane-bound calcium, activation of guanylate cyclase, increased cellular cyclic GMP and stimulation of enzyme secretion.

Potentiation of enzyme secretion from pancreatic acinar cells is observed with any agent which increases cyclic AMP (VIP or secretin) plus any agent which increases cyclic GMP (cholinergic agents or cholecystokinin) but not with the combination of two agents each of which increases the same cyclic nucleotide (e.g. VIP plus secretin or cholecystokinin plus carbamylcholine).

Dispersed gastric parietal cells remain functionally intact for at least 5 hours *in vitro*. In these cells gastrin and acetylcholine causes a significant increase in potassium influx while histamine and prostaglandins activate adenylate cyclase and increase cellular cyclic AMP.

Clinical studies of patients with Zollinger-Ellison syndrome indicate that the recently developed histamine $H_2$-receptor antagonist Cimetidine can be used to treat these patients. Our successful experience in eleven patients indicates that patients with Zollinger-Ellison syndrome need not be treated by total gastrectomy and that future therapeutic efforts will need to be directed toward antineoplastic chemotherapy.

Although the etiologic agent in the pancreatic cholera syndrome has not been identified unequivocally, patients with this syndrome can be treated by pancreatic resection and/or streptozotocin. The first patient treated with streptozotocin is alive and well four years after her initial therapy and has no evidence of tumor.

(Gardner, McCarthy, Batzri, Jensen, Peikin, Lopatin, Long, Uhlemann, Jackson, Sjodin, Tai, Conlon, Adams, Hahne, Rottman, Dyer, Garvey, Hyman, Costenbader)

Annual Report of the Section on Diseases of the Liver, DDB:NIAMDD

The Section on Diseases of the Liver is currently conducting six principal studies.

I. Studies of the Transport of Organic Anions by the Liver

A principal effort in this study involves the development of techniques for quantitating individual steps in the hepatic transport of various cholephilic anions, both in man and in animals, by employing kinetic studies with sulfobromophthalein (BSP), indocyanine green, cholyl-glycine and radiolabeled bilirubin. By means of kinetic techniques we have previously demonstrated that Gilbert's syndrome represents a heterogeneous condition. In type I subjects, the metabolic defect appears to be restricted to bilirubin metabolism. Type II subjects have, in addition, a defect in hepatic uptake of other cholephilic anions such as BSP and indocyanine green, whereas in type III subjects the initial hepatic uptake of these materials is normal but abnormal hepatic clearance results from an abnormality in a later stage in their transhepatic transport. Both fasting and post-prandial serum bile acid concentrations and cholyl-glycine disappearance curves have been shown to be entirely normal in all three of these subtypes of Gilbert's syndrome suggesting that the hepatic transport of bile acids occurs over separate pathways from those involved in the transport of other cholephilic anions. This conclusion has potential clinical significance in that the finding of a normal serum bile acid level or bile acid disappearance curve would mitigate strongly against the need for liver biopsy in the investigation of patients with an unconjugated hyperbilirubinemia.

To obtain new insights into the relationship between plasma bilirubin turnover and hepatic bilirubin clearance, studies of bilirubin kinetics were conducted in patients with hereditary spherocytosis before and after splenectomy. In patients whose pre-operative bilirubin clearance values were normal, the fall in bilirubin production resulting from splenectomy had no effect on hepatic bilirubin clearance, whereas in patients whose pre-operative bilirubin clearance values were reduced to values in the range found in Gilbert's syndrome there was a marked increase in hepatic bilirubin clearance following surgery. These findings suggest that in patients with Gilbert's syndrome the hepatic bilirubin transport system is operating at a level close to saturation and that bilirubin loading studies may represent a useful provacative test for the diagnosis of carriers of the Gilbert's syndrome gene who do not express the trait at basal levels of bilirubin production.

To assess the potential role of membrane carriers in the hepatic transport of organic anions the kinetics of binding of labeled BSP to canalicular and plasma membrane frations isolated from rat hepatocytes were studied. Saturable binding of BSP to both membranes was shown and this binding was competitively inhibited by bilirubin and indocyanine green but not by taurocholate. This investigation has provided further support for the hypothesis that organic anions other than bile salts share a common pathway for hepatic uptake and biliary excretion and that a membrane carrier protein may be involved in these processes.

The isolation of a pure preparation of biosynthetically labeled bilirubin conjugates in this laboratory has enabled kinetic studies of the hepatic metabolism of conjugated bilirubin to be carried out in rats. It has been shown that both hepatic uptake and net hepatic clearance of conjugated bilirubin is substantially faster than that of the unconjugated pigment, presumably due at least in part to the ability of the former pigment to bypass the conjugation step. In addition, the hepatic uptake of conjugated bilirubin, like that of the unconjugated pigment, was inhibited by other organic anions, such as BSP and indocyanine green, but not by glycocholate, suggesting that uptake is not mediated by simple diffusion but by a more complex mechanism shared with unconjugated bilirubin. In related studies storage of conjugated bilirubin within the liver cell was studied by analyzing labeled and unlabeled bile pigments in homogenates of rat liver made after the intravenous administration of $^{3}$H-labeled unconjugated bilirubin. The data indicated that bilirubin monoglucuronide but not bilirubin diglucuronide accumulates to an appreciable extent within the liver cell. In addition, conjugated bilirubin was found to have a high affinity for glutathione-S-transferases such as ligandin. Based on these findings it is suggested that microsomal monoconjugation of bilirubin may occur while it is bound to a glutathione-S-transferase and that the biliary excretion of bilirubin may involve interaction of a bilirubin monoglucuronide glutathione-S-transferase complex with the bile canalicular membrane with resultant excretion of the monoglucuronide or local formation and immediate excretion of the diglucuronide. (PD Berk, J Reichen, A Wolkoff, BL Blitzer, M Berman, J Waggoner, J Vergalla, J Ketley, WB Jakoby).

II. Immunologic Studies in Chronic Hepatitis and Primary Biliary Cirrhosis.

The role of abnormal immune mechanisms in the pathogenesis and perpetuation of the hepatobiliary lesions in chronic hepatitis and primary biliary cirrhosis (PBC) is being studied. Patients selected for study undergo a comprehensive evaluation of their immunologic status, which includes tests of the functional capacity of the afferent (antigen processing) and efferent (cellular and humoral) limbs of the immune response. Using an *in vitro* assay system which has been shown to be both sensitive and reproducible, the cytotoxic potential of lymphocytes has been studied using two different target cells: the Chang cell, a human liver-derived cell, and the EL-4 mouse sarcoma cell, a non-human non-hepatic cell.

In contrast to chronic hepatitis, spontaneous cell-mediated cytotoxicity (SCMC) of lymphocytes was shown to be defective against both target cells in PBC. No defect in mitogen-induced cytotoxicity (MICC) or antibody-dependent cytotoxicity (ADCC) was demonstrated in either disease. In both diseases SCMC and ADCC were shown to be mediated by a subpopulation of lymphocytes which lack surface immunoglobulin and bear Fc receptors whereas MICC was mediated by lymphocytes which lack both surface immunoglobulin and Fc receptors. Thus, a difference in cytotoxic function in PBC and chronic hepatitis has been defined in that the former but not the latter disease is associated with a defect in the spontaneous cytotoxic function of lymphocytes which lack surface immunoglobulin but bear Fc receptors.

The methods employed in these studies have enabled the question whether lymphocytes bearing complement receptors have cytotoxic potential to be evaluated in vitro. Starting with peripheral blood mononuclear cells it has been shown that depletion of complement receptor-bearing cells was associated with enrichment of Fc positive and surface immunoglobulin negative cells, depletion of phagocytes and surface immunoglobulin positive cells and normal or enchanced SCMC, MICC and ADCC. In contrast, depletion of Fc positive cells was associated with enrichment of complement receptor-bearing cells and virtually no capacity to induce SCMC or ADCC. These experiments indicate that at least the majority of cells which mediate SCMC, MICC and ADCC do not bear complement receptors.

Methods are being developed for the isolation of pure preparations of hepatocytes from animal livers and from biopsies of human livers for use as target cells in cytotoxicity assays.

The ability of the reticuloendothelial system to clear particles from the circulation by both nonspecific and immunospecific mechanisms has been studied in normal subjects and patients with PBC, chronic hepatitis and alcoholic cirrhosis. The clearance of both tracer and test doses of micro-aggregated albumin was normal in each instance indicating normal hepatic blood flow and a normal phagocytic capacity of the reticuloendothelial system in every subject studied. The clearance and subsequent phagocytosis of IgG-coated radiochromated autologous red cells, which depends on Fc receptor-bearing fixed macrophages in the spleen was normal in all groups, except patients with PBC, the majority of whom had an increased rate of clearance. This observation may imply activation of the reticuloendothelial system in this disease. In contrast, the clearance of IgM-coated radio-chromated autologous red cells was markedly decreased in each patient with PBC but was normal in all of the other subjects studied. IgM-coated cells rapidly become coated with C3b in the circulation and their clearance is mediated by C3b-receptor bearing Kupffer cells in the liver, following which they either undergo phagocytosis or are released back into the circulation as C3d-coated cells as a result of the action of circulating C3b inactivator. To obtain further insight into this clearance defect in PBC, a compartmental model of the C3b clearance process was developed. Using computer simulations the model was able to account for the observed data and showed that the major determinant of the defect was a reduced initial rate of removal of C3b-coated cells from the circulation. It was shown that the defect could not be attributed to a deficiency of components of the classical complement pathway, to increased or decreased activity of C3b-inactivator, or to competitive inhibition of C3b receptors on Kupffer cells by soluble immune complexes. However, the defect could be due to a decrease in the number or affinity of C3b-receptors or to partial saturation of these receptors by excess circulating C3b in PBC. (EA Jones, JM Vierling, CJ Steer, SP James, W Strober [NCI], DL Nelson [NCI], CJ Jaffe [NIAID], MM Frank [NIAID]).

III. Studies of Copper Metabolism in Man Using $^{67}Cu$ and $^{64}Cu$

Accumulation of copper in the liver may contribute to hepatic injury. To study the nature of the abnormalities of copper metabolism in hepatic

copper overload states such as Wilson's disease and primary biliary cirrhosis, the fate of simultaneously administered $^{67}Cu$ and $^{64}Cu$ is being followed in patients with these diseases and normal subjects. One of the isotopes is given orally and the other intravenously. Serial measurements are made of radioactivity due to both isotopes in the whole body, blood, urine, feces and, using an external probe, the liver. Data obtained from blood comprise separate determinations of radioactivity in red cells, ceruloplasmin and ceruloplasmin-free plasma. Analysis of experimental data, which has involved the use of a computer to fit, deconvolute and integrate plasma radiocopper curves has indicated that (i) a pool of nonceruloplasmin-bound copper which rapidly exchanges with plasma nonceruloplasmin-bound copper exists in control subjects but is either saturated or absent in patients with PBC, (ii) about 50% of orally administered radiocopper is absorbed in both normal subjects and patients with PBC, (iii) in the presence of a positive copper balance in PBC, the degree of intestinal absorption and hepatic extraction of orally-administered radiocopper is inappropriate, (iv) a major defect in copper metabolism in PBC is decreased biliary excretion of copper, (v) there is a decreased fractional rate of uptake of copper by the liver in Wilson's disease which is not attributable solely to an increased hepatic copper concentration, (vi) the incorporation of radiocopper into ceruloplasmin can be used to differentiate between two hepatic copper overload states, incorporation being negligible in Wilson's disease and appreciable in PBC, and (vii) incorporation of $^{67}Cu$ into ceruloplasmin is almost linear for at least 7 days after its administration to both normal subjects and patients with hepatic copper overload suggesting rapid equilibration of $^{67}Cu$ with a large hepatic pool of copper which has a slow turnover rate and a loss pathway with a small fractional transfer rate to the precursor pool of copper for ceruloplasmin synthesis. (EA Jones, JM Vierling, R Shrager [CR], WF Rumble [CC], R Aamodt [CC]).

IV. Studies of the Metabolism of Radioiodinated Alpha-1-Antitrypsin and Desialylated Alpha-1-Antitrypsin in Man.

Homozygous alpha-1-antitrypsin ($\alpha_1$-AT) deficiency (phenotype PiZZ) may be associated with cholestasis in the neonatal period, cirrhosis, liver cell carcinoma and emphysema. In this condition hepatocytes have been shown to contain globules of $\alpha_1$-AT-like material devoid of sialic acid, and the $\alpha_1$-AT in plasma, which is present in abnormally low concentration, has also been shown to have a reduced content of sialic acid. The aim of these studies is to investigate $\alpha_1$-AT metabolism in normal subjects and patients with $\alpha_1$-AT deficiency and, in particular, to examine the relationship between the molecular structure of $\alpha_1$-AT and the rate of catabolism of this protein. Using normal plasma from individuals of phenotype PiMM, immunochemically pure $\alpha_1$-AT has been isolated and shown to have retained its characteristic microheterogeneity when subjected to acid starch gel electrophoresis and its ability to inhibit trypsin. This material after trace-labeling with radio-iodine has been used to study the turnover of $\alpha_1$-AT in normal (phenotype PiMM) volunteers. The serum concentration of $\alpha_1$-AT was shown to vary independently of its fractional catabolic rate but correlated with the synthetic rate. Analysis of the turnover data indicated that $\alpha_1$-AT is catabolized in, or in close relationship to, the intravascular compartment. Analogous studies in normal volunteers were performed after desialylating $\alpha_1$-AT with neuraminidase.

The desialylated protein was shown to be removed from plasma approximately 150 times faster than the intact protein. Its rapid disappearance from plasma was associated with an equally rapid uptake of the radioiodine label by the liver. This finding emphasizes that the catabolism of $\alpha_1$-AT can be influenced by the molecular structure of its carbohydrate moiety and suggests the existence of hepatic receptors for desialylated glycoproteins in the human liver. The administration of labeled intact and desialylated (PiMM) $\alpha_1$-AT to patients with $\alpha_1$-AT deficiency (phenotype PiZZ) and the administration of labeled $\alpha_1$-AT isolated from patients with $\alpha_1$-AT deficiency to both normal subjects and patients with $\alpha_1$-AT deficiency will enable the contribution of decreased hepatic release of $\alpha_1$-AT and increased hepatic uptake of $\alpha_1$-AT to the low serum concentration of $\alpha_1$-AT in $\alpha_1$-AT deficiency to be assessed. Whether $\alpha_1$-AT that is biosynthetically deficient in sialic acid behaves differently from that which is deficient in sialic acid as a result of neuraminidase treatment will also be determined by these studies. (EA Jones, J Vergalla, CJ Steer, JM Vierling, PR Bradley-Moore [CC]).

V. Immunologic Properties of the Hepatic Asialoglycoprotein-Binding Protein

A glycoprotein isolated from rabbit hepatocytes which has the ability to bind specifically desialylated glycoproteins has recently been shown to be capable of inducing mitogenesis in human desialylated lymphocytes. The immunologic properties of this hepatic protein, the first lectin of mammalian origin to be recognized, has been studied further. It has been shown that the isolated hepatic binding protein has the ability to increase the cytotoxicity of human lymphocytes against Chang target cells to the same degree as the plant lectin phytohemagglutinin. Hepatic binding protein did not increase the cytotoxicity of intact lymphocytes. In addition, it was shown that desialylated lymphocytes induced significantly more cytotoxicity in isolated rabbit hepatocytes, which bear hepatic binding protein on their surface, than did intact lymphocytes, and that this phenomenon could be blocked by asialo-orosomucoid (a desialylated glycoprotein) but not by intact orosomucoid. These findings suggest that the cytotoxicity induced by membrane-bound hepatic binding protein is similar in mechanism to that induced by the isolated protein and that the induced cytotoxicity probably depends on the interaction between hepatic binding protein and exposed galactose residues in desialylated lymphocytes. To evaluate the full significance of these findings it is necessary to relate them to the observations that desialylated lymphocytes are preferentially sequestered in the liver of rats and that myxoviruses and other human pathogens that possess neuraminidase are potentially capable of desialylating lymphocytes in vivo. If the hepatic sequestration of desialylated lymphocytes is mediated by hepatic binding protein it is likely that hepatic binding protein on the surface of Kupffer cells rather than on the surface of hepatocytes would be involved and it is conceivable that local cellular injury could ensue. Alternatively, it is possible that desialylated lymphocytes become coated with IgG or IgM molecules in the circulation and that their hepatic sequestration is mediated by immunospecific binding to Fc or C'3 receptors on Kupffer cells. Further studies are in progress to determine whether hepatic binding protein exists on the surface of Kupffer cells and to determine the mechanism and possible pathophysiologic significance of

hepatic clearance of desialylated lymphocytes. The interaction of hepatic binding protein with desialylated lymphocytes may be analogous to processes which cause lymphocytes to become cytopathogenic in human disease. (EA Jones, JM Vierling, CJ Steer, JW Hickman).

VI. The Use of Artificial Hepatic Support Devices for the Treatment of Fulminant Hepatic Failure.

An animal model of acute hepatic failure has been developed with a view to testing the effectiveness of hemoperfusion through albumin-agarose affinity chromatography columns as a means of providing temporary hepatic support. The model was induced in an inbred strain of rabbits by administering intravenously a precise dose of the selective hepatotoxin galactosamine-HCl. The model induced has the advantages of being (i) non-hazardous to personnel, (ii) fairly reproducible, (iii) potentially reversible and (iv) closely resembling human fulminant hepatic failure in its clinical, electroencephalographic, serum biochemical and hepatic histologic features.

Arousal of patients from coma due to fulminant hepatic failure may be attributable at least in part to depletion of protein-bound substances toxic to the brain from a slowly exchanging extravascular pool such as the brain or CSF. The hypothetical effects of different hemoperfusion schedules on the kinetics of depletion of a protein-bound substance, unconjugated bilirubin, were simulated using a computer. Data on the kinetics of unconjugated bilirubin in man and experimentally determined values for the efficiency of hemoperfusion through albumin-agarose columns in removing unconjugated bilirubin from plasma were used in the simulations. The results suggest that simple alterations in the schedule of hemoperfusions might accelerate depletion of toxins from an extravascular pool and hence promote earlier arousal and possibly more frequent survival in patients with fulminant hepatic failure. (PD Berk, BL Blitzer, JG Waggoner, EA Jones, H Gralnick [CC], D Towne [CH], J Butler [CH], V Weise [M], I Kopin [M], I Walters [N], P Teychenne [N], D Goodman [C].

ANNUAL REPORT SUMMARY
DIGESTIVE DISEASES BRANCH

Phoenix Clinical Research Section

The investigations performed by the Section concern metabolic diseases that are particularly prevalent among American Indians. Most of the investigations stem from the epidemiological investigations carried out by the Southwest Field Studies Section of the Epidemiology and Field Studies Branch, and cluster in three general areas, namely; gallbladder disease, lipid metabolism, and diabetes mellitus.

Gallbladder disease: Investigations of biliary lipids in the fasting state were undertaken to determine why bile exhibits increased lithogenicity during fasting. While bile acid pool size remained essentially unchanged, bile acid synthesis and the fractional catabolic rate of cholic acid was found to be reduced to approximately a third the rate observed in the nonfasting state. Concomitantly, however, the molar percent cholesterol was also reduced.

The effects of long-term administration of phenobarbital and the effects of a high fiber diet, both purported to result in a reduction in biliary lithogenicity, were investigated in normal subjects. No beneficial effect on biliary lipid composition of either of these proposed therapeutic modalities was found.

The changes in the lipid composition of gallbladder bile and bile acid pool size have been determined in Pima Indians in the teenage years to determine the age at which the bile of Pima Indians becomes lithogenic. These data will help to determine the factors which relate to the extraordinarily high prevalence of cholesterol gallstone formation which occurs in Pima females between 20 and 30 years of age. The relationships of bile acid pool size and biliary lithogenicity to age and sex hormone levels have been determined in an attempt to identify the reasons for increased lithogenicity which was found in the teenage years.

To determine the mechanisms of cholesterol gallstone formation the effects of a known lithogenic diet on gallstone formation in hamsters has been investigated by the determination of HMG CoA reductase activity. This is the rate limiting enzyme in the synthesis of cholesterol and investigations completed this year have shown that HMG CoA reductase activity increases considerably when hamsters are placed on the lithogenic diet indicating that a major effect of the lithogenic diet is to induce cholesterol gallstone formation by increasing the rate of cholesterol synthesis.

Lipid Metabolism: As southwestern American Indians have low serum cholesterol levels and extraordinarily low prevalences of coronary artery disease compared to Caucasians, even in spite of a high prevalence of diabetes, the cholesterol carrying lipoprotein fractions have been investigated in Southwestern American Indians and Caucasians. Low levels of low density lipoprotein cholesterol appear to characterize the American Indian.

Investigations of LDL turnover have now been completed which indicate that the absolute synthetic rate and total pool of LDL apoprotein was significantly lower in the Indians than in the Caucasians. In the same investigation it was found that HDL lipoprotein cholesterol levels were at least as high and possibly higher than those of Caucasian controls.

As recent studies have implicated high density lipoprotein cholesterol as a negative independent risk factor in the genesis of atherosclerosis, as investigation has been initiated to determine the lipoprotein composition in both Indians and Caucasians, and the fractions of cholesterol and triglyceride associated with each fraction of the lipoprotein subclasses, as isolated by ultracentrifugation. Determinations are being performed in normal and diabetic Indians, with and without coronary disease, and Caucasian controls. From this study, it should be possible to determine if diabetes is associated with changes in the serum lipoproteins, and to delineate further the importance of HDL cholesterol in the pathogenesis of ischemic heart disease and to determine whether or not the relative infrequency of coronary artery disease among the Southwestern Indians, is attributable to differences in the proportions of HDL and LDL cholesterol previously found.

Diabetes Mellitus. In conjunction with the Southwest Field Studies Section of the Epidemiology and Field Studies Branch, diabetes mellitus which occurs in extraordinarily high prevalence in many southwestern American Indian tribes, and, in particular, among the Pima Indians, has become the primary area of investigation of the section. The effect of uncontrolled hyperglycemia on lipid metabolism has been investigated. Cholesterol synthesis and bile acid excretion were enhanced in the uncontrolled hyperglycemic state, as was the bile acid pool size. Control of the hyperglycemia resulted in the fall in plasma cholesterol and plasma triglyceride levels, and a reduction in cholesterol synthesis and bile acid pool size that was associated with a significant increase in the lithogenicity of gallbladder bile. Examination of lipoprotein levels indicated that the low density lipoprotein cholesterol level was the fraction reduced most by control of the hyperglycemia. These findings may have important practical applications in the management of diabetes mellitus.

It is well known that weight reduction can effect a significant improvement in glucose tolerance in subjects with adult onset diabetes. The mechanisms of this improvement however, have been incompletely investigated. The effects of weight reduction on glucose tolerance, insulin secretion, glucagon secretion, and insulin receptor concentrations have been determined among Pima Indians with recent onset symptomatic fasting hyperglycemia. To the present time, normalization of the fasting blood sugar level has been achieved in all subjects and is accompanied by a major increase in insulin secretory responses to glucose stimuli. However, the insulin responsiveness of these subjects to an arginine infusion before and after control of the hyperglycemia changes little. These findings indicate that a major abnormality in recent onset adult diabetes is a failure of the pancreas to respond to glucose stimuli, whereas the ability to manufacture and secrete insulin in response to amino acid stimuli remains essentially normal. These findings suggest that a precipitating factor in

maturity onset diabetes is a failure of the glucose sensing mechanism of the pancreas. It is hoped that extension of these studies will result in the better understanding of the factors which result in the precepitation of maturity onset diabetes among those genetically predisposed to the disease. A clear understanding of such a mechanism, might ultimately lead to the prevention of decompensation of glucose tolerance among those predisposed.

Additional investigations to determine the nature of the basic lesion which predisposes to diabetes have been conducted among selected groups of Pima Indians, including those most likely to develop diabetes, the genetic prediabetic, and both Pima Indian and Caucasian controls. The investigations have included studies of gluco-regulatory hormones, muscle capillary basement membrane thickness, and urinary protein excretion in these groups and in recent onset Pima Indian diabetics. No evidence of differences in the pattern of insulin response to oral and intravenous glucose, or glucagon response to intravenous arginine or glucose have been found amongst the genetic prediabetic. Recent onset Pima Indian diabetics, however, have abnormalities in these responses to all of these stimuli, which are qualitatively the same as those described in Caucasians with maturity onset diabetes.

Clinical investigations of the muscle capillary basement membrane thickness in these same patient groups have been completed and are presently being analyzed. The results to date indicate that the diabetics have thickening of the capillary basement membrane, when measured by either the Siperstein or Williamson techniques, but that the proportion with thickened capillary basement membranes is greater with the Siperstein method. Among the prediabetics, some evidence of capillary basement membrane thickening prior to the onset of glucose intolerance has been found by the Siperstein method, primarily among the older prediabetics. Observations amongst older prediabetics and genetically normal Pima Indians have been extended and the results will be available shortly.

The excretion of urinary macromolecular proteins has also been examined in each of these four patient groups to determine whether detailed measurements provide a sensitive index of the presence of renal disease prior to the onset of clinical proteinuria, as determined by conventional measures. While no changes were observed in the normal and prediabetic Indians and Caucasians, it was found that a number of proteins with a molecular weight ranging from 44,000 to 150,000 daltons were excreted in excessive concentrations by diabetics of documented recent onset. These findings suggest that changes in the renal-vascular permeability of these proteins occur very early in the course of diabetes and suggest that it may be possible to predict which diabetics will ultimately develop clinically important proteinuria or renal failure. These studies will be continued among the Pima Indian population to determine whether these measures do indeed have prognostic significance. If so, urinary macromolecular protein measurements will provide an important non-invasive investigative procedure for diabetic nephropathy.

Previous studies have indicated that the Pima Indian has excessive and, as yet, unexplained hyperinsulinemia. Understanding the mechanisms of this hyperinsulinemia may prove to be important in determining why the Pima Indian is likely to develop diabetes mellitus. The hyperinsulinemia may be the result of an intracellular abnormality, an insulin receptor abnormality, a circulating antireceptor antibody or antiinsulin antibody or, possibly, a structural abnormality of Pima insulin per se. In order to investigate these possibilities a number of studies have been initiated of insulin receptors in circulating monocytes and fibroblast cultures. The fibroblast culture is also being utilized to determine parameters of glucose transport and metabolism. To the present time, no evidence of a reduced number of insulin receptors or abnormal affinity for insulin has been found, but an apparent abnormality in the down-regulation of insulin receptors by circulating fasting insulin has been identified. Such an abnormality may, however, be a manifestation of an intracellular abnormality, rather than an abnormality of the receptor per se. In fibroblast cultures of Caucasians and Pima Indians both diabetic and nondiabetic, glucose uptake has been measured in the presence and absence of insulin and uptake has been shown to occur within the range of physiologic levels of insulin. The parameters of growth of fibroblasts derived from prediabetic, normal and diabetic Pima Indians, including investigations of plating efficiency, population doubling, confluent cell density and total number of population doublings are being performed. Such investigations should yield important information on whether the possible genetic defects in adult onset diabetes are reflected in altered parameters of cell growth and glucose metabolism.

| SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space) | U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT** | PROJECT NUMBER Z01 AM 53001-07 DDB |
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PERIOD COVERED

July 1, 1976 to Sept 30, 1977

TITLE OF PROJECT (80 characters or less)

Studies of Membrane Function

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

| | | | |
|---|---|---|---|
| PI: | J. D. Gardner | Chief, Section on Gastroenterology | DDB, NIAMDD |
| OTHERS: | M. Jackson | Guest Worker | DDB, NIAMDD |
| | L. Sjodin | Guest Worker | DDB, NIAMDD |
| | S. Batzri | Visiting Associate | DDB, NIAMDD |
| | E. R. Uhlemann | Clinical Associate | DDB, NIAMDD |
| | R. T. Jensen | Guest Worker | DDB, NIAMDD |
| | B. Tai | Guest Worker | DDB, NIAMDD |
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| | P. Hyman | Clinical Associate | DDB, NIAMDD |
| | C. Costenbader | Technician | DDB, NIAMDD |

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LAB/BRANCH

Digestive Diseases Branch

SECTION

Section on Gastroenterology

INSTITUTE AND LOCATION

NIAMDD, NIH, Bethesda, Maryland 20014

| TOTAL MANYEARS: | PROFESSIONAL: | OTHER: |
|---|---|---|
| 3.0 | 1.6 | 1.6 |

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The broad categories which are included in the project are: 1) Characterizing functionally the mechanism by which various substrates cross the plasma membrane of different mammalian cells; 2) identifying the metabolic and humoral factors which influence the transport of various substrates across the plasma membrane; 3) developing techniques which will distinguish between binding of a substrate to the membrane and translocation of the substrate across the membrane; 4) characterizing the mechanism by which the membrane transport of various substrates is altered in certain diseases; and 5) relating these alterations of membrane transport to the pathogenesis and clinical manifestations of the disease.

By increasing cellular calcium in thymic lymphocytes one can abolish the stimulation of amino acid influx caused by cholera toxin, prostaglandin, theophylline, exogenous cyclic AMP or insulin. Ethanol will abolish the stimulation of amino acid influx caused by insulin but does not alter stimulation caused by exogenous or endogenous cyclic AMP.

Publications:

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| SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space) | U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT** | PROJECT NUMBER ZO1 AM 53002-05 DDB |
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PERIOD COVERED
July 1, 1976 to Sept 30, 1977

TITLE OF PROJECT (80 characters or less)
Gastrointestinal Hormones

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

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LAB/BRANCH
Digestive Diseases Branch

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| TOTAL MANYEARS: | PROFESSIONAL: | OTHER: |
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| 4.0 | 2.0 | 2.0 |

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

In vitro systems are being used to study the mechanism of action of gastrin, secretin, cholecystokinin and vasoactive intestinal peptide with their specific membrane receptors.

Clinical investigations are directed toward developing alternative forms of therapy for and elucidating the pathogenesis of disorders characterized by ectopic production of gastrointestinal hormones (e.g., Zollinger-Ellison syndrome and pancreatic cholera).



PHS-6040

In dispersed acinar cells from guinea pig pancreas, VIP and secretin interact with a common membrane receptor, activate adenylate cyclase and increase cellular cyclic AMP. In these same cells cholecystokinin and cholinergic agents cause release of membrane-bound clacium, increased cellular cyclic GMP and stimulation of amylase secretion.

In patients with Zollinger-Ellison syndrome the histamine $H_2$-blocking agent, Cimetidine, will abolish gastric acid secretion and, as a result, is a therapeutic alternative to total gastrectomy.

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| SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space) | U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT | PROJECT NUMBER Z01 AM 53003-04 DDB |
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PERIOD COVERED

July 1, 1976 to Sept 30, 1977

TITLE OF PROJECT (80 characters or less)

Gastric Emptying

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

| | | | |
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| | M. Berman | Chief, Lab. of Theoretical Biology | LTB NCI |

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GI Clinic, National Naval Medical Center

LAB/BRANCH

Digestive Diseases Branch

SECTION

Section on Gastroenterology

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NIAMDD, NIH, Bethesda, Maryland 20014

| TOTAL MANYEARS: | PROFESSIONAL: | OTHER: |
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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Using a dye dilution technique combined with rapid sampling, gastric emptying and secretion are being evaluated in vivo in man, dogs, and monkeys. Results obtained from such studies are being used to develop a mathematical model of gastric emptying and to explore the role played by gastric emptying in various clinical disorders such as postoperative ileus, Zollinger-Ellison syndrome, and duodenal ulcer.

Patients with Zollinger-Ellison syndrome have increased fractional rate of gastric emptying both during fasting and following a meal. In contrast, administration of pentagastrin to normal subjects does not alter fractional rate of emptying during fasting and slows the rate of fractional emptying after a meal. These results suggest that the increased rate of fractional gastric emptying seen in patients with Zollinger-Ellison syndrome is not attributable to gastrin but may be due to an undefined neural or humoral factor.

Publications:

Dubois, A., Van Eerdewegh, P., Line, B., Van Maele, P., and Gardner, J. D.: A new method for simultaneous determination of gastric emptying and secretion. Proc. 5th Int. Symp. on Gastrointestinal Motility, Van Trappen, Ed., Leuven, 1977, pp. 244-247.

Dubois, A., Van Eerdewegh, P., and Gardner, J. D.: Gastric emptying and secretion in Zollinger-Ellison Syndrome. J. Clin. Invest. 59:255-263, 1977.

| SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space) | U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT | PROJECT NUMBER Z01 AM 53004-05 DDB |
|---|---|---|

PERIOD COVERED
July 1, 1976 to Sept 30, 1977

TITLE OF PROJECT (80 characters or less)
Cyclic Nucleotide Mediated Functions

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

| | | | |
|---|---|---|---|
| PI: | J. D. Gardner | Chief, Section on Gastroenterology | DDB NIAMDD |
| OTHER: | M. Jackson | Guest Worker | DDB NIAMDD |
| | L. Sjodin | Guest Worker | DDB NIAMDD |
| | S. Batzri | Visiting Associate | DDB NIAMDD |
| | B. W. Long | Clinical Associate | DDB NIAMDD |
| | S. R. Peikin | Clinical Associate | DDB NIAMDD |
| | R. N. Lopatin | Clinical Associate | DDB NIAMDD |
| | R. T. Jensen | Guest Worker | DDB NIAMDD |
| | B. Tai | Guest Worker | DDB NIAMDD |
| | P. Hyman | Clinical Associate | DDB NIAMDD |
| | C. Costenbader | Technician | DDB NIAMDD |

COOPERATING UNITS (if any)

LAB/BRANCH
Digestive Diseases Branch

SECTION
Section on Gastroenterology

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20014

| TOTAL MANYEARS: | PROFESSIONAL: | OTHER: |
|---|---|---|
| 4.0 | 2.0 | 2.0 |

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS (b) HUMAN TISSUES (c) VERTEBRATES

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

In vitro systems are being used to characterize the mechanism by which cyclic nucleotides alter cell function and to explore the mechanism of action of agents whose effect on cell function is mediated by cellular accumulation of cyclic nucleotides.

In dispersed acinar cells prepared from guinea pig pancreas the activity of cyclic nucleotide phosphodiesterase is intimately dependent on cellular calcium concentration. By varying the extracellular calcium concentration one can manipulate cellular concentrations of both cyclic AMP and cyclic GMP, and by so doing modify the final hormone response of the tissue.

Publications:

Gardner, J. D., Aurbach, G. D., Spiegel, A. M. and Brown, M. E.: Receptor function and ion transport in turkey erythrocytes. In Greep, R. O. (Ed.): Recent Progress in Hormone Research, Vol. 32, Proceedings of the 1975 Laurentian Hormone Conference, New York, Academic Press, pp 567-595, 1976.

Aurbach, G. D., Brown, E. M., Spiegel, A. M. and Gardner, J. D.: Beta-adrenergic receptors, cyclic AMP and ion transport in the avian erythrocyte. In Beers, R. F. Jr. and Bassett, E. G. (Eds.): Cell Metabolism Receptors for Viruses, Antigens and Antibodies, Polypeptide Hormones, and Small Molecules. Proceeds of the 9th Miles International Symposium, New York, Raven Press. pp. 379-392, 1976.

Brown, E. M., Gardner, J. D. and Aurbach, G. D.: Direct determination of ligand interactions with beta-adrenergic receptors on intact turkey erythrocytes: correlation of binding with biological activity. Endocrinology, 99: 1369-1376, 1976.

Waldman, D. B., Gardner, J. D., Zfass, A. M., and Makhlouf, G. M.: Effects of vasoactive intestinal peptide (VIP), secretin and related peptides on colonic transport and adenylate cyclase activity. Gastroenterology (In Press).

| SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space) | U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT | PROJECT NUMBER Z01 AM 53005-03 DDB |
|---|---|---|

PERIOD COVERED
July 1, 1976 to Sept. 30, 1977

TITLE OF PROJECT (80 characters or less)
Biochemistry of the Small Intestinal Mucosa

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

| | | | |
|---|---|---|---|
| PI: | D. M. McCarthy | Visiting Scientist | DDB NIAMDD |
| OTHER: | R. J. May | Clinical Associate | DDB NIAMDD |
| | J. D. Gardner, | Chief, Section on Gastroenterology | DDB, NIAMDD |

COOPERATING UNITS (if any)

LAB/BRANCH
Digestive Diseases Branch

SECTION
Section on Gastroenterology

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20014

| TOTAL MAN-YEARS: | PROFESSIONAL: | OTHER: |
|---|---|---|
| 1.6 | 1.0 | .6 |

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

This project was discontinued July 1, 1976. No further work has been done.

Project discontinued July 1, 1976

Publications:

May, R. J. and McCarthy, D. M.: Particulate' Hexokinase Activity in Rat Intestine. The Comparative Contributions of Mitochondria and Brush-Border Membranes. Biochem. J., 159:189-192, 1976.

| SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space) | U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT | PROJECT NUMBER Z01 AM 53,500-04 DDB |
|---|---|---|

PERIOD COVERED
July 1, 1976 through September 30, 1977

TITLE OF PROJECT (80 characters or less)
Studies of the Transport of Organic Anions by the Liver

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

| | | | |
|---|---|---|---|
| P.I.: | P. D. Berk | Chief, Sec. on Dis. of the Liver | DDB:NIAMDD |
| Other: | B. L. Blitzer | Clinical Associate | DDB:NIAMDD |
| | J. M. Vierling | Clinical Associate | DDB:NIAMDD |
| | M. D. Berman | Clinical Associate | DDB:NIAMDD |
| | J. Reichen | Guest Worker | DDB:NIAMDD |
| | W. B. Jakoby | Chief, Sec. on Enzyme and Cell Biol. | LBM:NIAMDD |

COOPERATING UNITS (if any)

None

LAB/BRANCH
Digestive Diseases Branch

SECTION
Section on Diseases of the Liver

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20014

| TOTAL MANYEARS: | PROFESSIONAL: | OTHER: |
|---|---|---|
| 4 | 3 | 1 |

CHECK APPROPRIATE BOX(ES)

[X] (a) HUMAN SUBJECTS [X] (b) HUMAN TISSUES [ ] (c) NEITHER

[ ] (a1) MINORS [ ] (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Plasma disappearance curves of radiolabeled bilirubin, sulfobromophthalein, indocyanine green and bile acids are obtained in normal volunteers, patients with hepatic disease and experimental animals. Curves are analyzed by computer to calculate anion clearance and, in the case of bilirubin, daily bilirubin turnover. Data are further analyzed in terms of compartmental models to estimate hepatic uptake and conjugation rates, storage capacity, liver:plasma concentration gradients and other parameters of the hepatic organic anion transport mechanism which are of interest both for physiologic studies and in the diagnostic classification of hepatic dysfunction.

Studies of the hepatic metabolism of conjugated bilirubin have been facilitated by the isolation of pure radiolabeled conjugates of bilirubin. An appreciable affinity of conjugated bilirubin for intrahepatic binding proteins, such as ligandin, and the existence of a significant intrahepatic storage pool for conjugated bilirubin have been demonstrated. These two observatons make a re-examination of current concepts of the nature of the hepatic excretion of conjugated bilirubin necessary.

Project Description:

Objectives:

1) To study the transport of organic anions from blood through the liver into bile, in order to characterize the biophysical nature of each of the transport steps involved in the overall hepatic excretory system.

2) To develop a capability for quantitating the function of each of these steps in patients with hepatic dysfunction by analysis of kinetic studies of various test substances such as sulfobromophthalein, indocyanine green and radiolabeled bilirubin.

Methods Employed:

Unconjugated bilirubin-$^{3}H$ or -$^{14}C$ is produced biosynthetically in dogs with external biliary fistulae using δ-aminolevulinic acid-$^{14}C$ or -$^{3}H$ as a precursor. After appropriate crystallization and filtration to render the product sterile and pyrogen-free, radiolabeled bilirubin is injected intravenously into patients or animals in order to determine plasma radiobilirubin disappearance curves. Analysis of these curves permits direct calculation of both hepatic bilirbuin clearance ($C_{BR}$: ml/min/kg) and plasma bilirubin turnover (BRT: μmols/kg/day). Further analysis of the disappearance curves in terms of a previously proposed compartmental model of bilirubin metabolism permits direct calculation of such parameters of physiologic interest as hepatic bilirubin uptake rate, the intrahepatic bilirubin concentration, the liver to plasma concentration gradient and the hepatic bilirubin storage capacity. Similar kinetic analyses of plasma disappearance curves of sulfobromophthalein and indocyanine green are also performed.

Major Findings:

By means of kinetic techniques we have previously demonstrated that Gilbert's syndrome is a heterogeneous condition. In type I the metabolic defect appears to be restricted to bilirubin metabolism. Type II has, in addition, a defect in the hepatic uptake of cholephilic anions such as BSP and indocyanine green, whereas in type III the initial hepatic uptake of these materials is normal but abnormal hepatic clearance results from a defect in a later stage in their transhepatic transport. The presence of an abnormal BSP test in the latter two groups often leads to considerable confusion and frequently necessitates a percutaneous liver biopsy to rule out the existence of structural liver disease. During the past two years investigation of bile acid metabolism in Gilbert's syndrome has demonstrated that fasting and postpranial serum bile acid concentrations and cholylglycine disappearance studies are entirely normal in all three of these subtypes of Gilbert's syndrome. These studies confirm previously fragmentary information which suggests that the transhepatic transport of bile acids occurs over separate pathways from those involved in the excretion of other cholephilic anions. This finding is also of clinical significance in that even minimal structural hepatic disease has been found almost invariably to be associated with an abnormality in either the postpranial bile acid

concentration or plasma bile acid disappearance rates. Hence, in those subgroups of Gilbert's syndrome associated with abnormalities of BSP metabolism the presence of normal bile acid studies strongly mitigates against the existence of structural liver disease and can be used as a means of obviating the necessity for invasive procedures such as liver biopsy in these patients.

Studies of the relationship of the rate of bilirubin production to the efficiency of hepatic bilirubin clearance have been continued using patients with hereditary spherocytosis as the study population. Since splenectomy essentially abolishes the hemolytic anemia in this condition, studies of bilirubin kinetics before and after splenectomy enable the evaluation of hepatic function in a given individual at two different rates of bilirubin production without any other exogenous or extraneous influences such as the administration of drug therapy. To date, plasma bilirubin turnover and hepatic bilirubin clearance have been determined pre- and post-splenectomy in 13 patients with hereditary spherocytosis in whom a significant hemolytic state was manifested by a pre-operative chromium-51 red cell half life of 12 $\pm$ 2 days. In five of the patients (group A) pre-operative hepatic bilirubin clearance was normal (0.58 $\pm$ 0.10 ml/min/kg) and was unchanged post-operatively despite a marked fall in plasma bilirubin turnover from 18 $\pm$ 5 to down to 5 $\pm$ 2 mg/kg/day. In the other 6 patients (group B) pre-operative hepatic bilirubin clearance was reduced to a range suggestive of Gilbert's syndrome (0.12-0.28 ml/min/kg) but increased to 176 $\pm$ 23% of baseline ($p < .01$) as bilirubin turnover fell from 21 $\pm$ 4 to 6 $\pm$ 1 mg/kg/day post-operatively. In 3 of the 6 patients in group B, post-operative values for hepatic bilirubin clearance were within the normal range and in the remainder they were just below the normal range. Nevertheless, family history and studies of the bilirubin conjugating enzyme, UDP glucuronyl transferase, performed on needle biopsy specimens obtained during surgery, suggested that these patients did, in fact, have Gilbert's syndrome. These studies are compatible with the concept that some aspect of bilirubin transport is more readily saturated by an increased bilirubin load in patients with Gilbert's syndrome than in normal subjects. Furthermore, latent Gilbert's syndrome which is not evident at basal rates of bilirubin production may be unmasked by situations, such as hemolytic states, which present an increased bilirubin load to the liver. These studies may explain why family studies in Gilbert's syndrome which are based solely on measurements of plasma bilirubin concentration almost invariably detect a smaller number of affected family members in a given kindred than would be predicted by the expected autosomal dominant pattern of inheritance. As a prelude to future family studies, we are currently studying the kinetics of an injected load of 5 mg/kg of bilirubin in normal volunteers and patients with each of the three subtypes of Gilbert's syndrome. Preliminary data suggests that in both patients and normal volunteers the hepatic clearance of a 5 mg/kg load of bilirubin is less than that of a tracer dose of radiolabeled material. However, in patients with Gilbert's syndrome the reduction produced by the 5 mg/kg load is proportionally much greater than that observed in normal volunteers. If this observation is confirmed in a larger series, then it may be possible to use the disappearance curve of a 5 mg/kg loading dose of bilirubin as a more effective screening test for latent Gilbert's syndrome in genetic studies.

Previous animal studies based on crude and highly impure preparations of conjugated bilirubin have suggested that bilirubin conjugates did not bind to any significant degree to intracellular binding proteins, such as ligandin. Accordingly, it was widely believed that bilirubin was rapidly excreted from the liver cell almost immediately after conjugation. Recent studies by Dr. William Jakoby (A:LBM) using highly purified preparations of conjugated bilirubin furnished from this department have, in fact, indicated that there is a high affinity of conjugated bilirubin for the various intracellular binding proteins (glutathione-S-transferases) and that the affinity of ligandin for conjugated bilirubin approximates that for the unconjugated pigment. Based on these observations detailed studies of the transhepatic transport of conjugated bilirubin have been carried out in rats. Quantitative kinetic analyses of the data provides clearcut evidence that there is, in fact, a significant pool of protein bound conjugated bilirubin within the liver cell analagous to the "storage" of unconjugated material bound to similar proteins. Preliminary data suggest that the pool consists largely of bilirubin monoglucuronide with a relatively small content of bilirubin diglucuronide. This observation may be of relevance to recently reported observations that bilirubin diglucuronide is not formed by the microsomal enzyme bilirubin glucuronyl transferase but is, in fact, the result of a transesterification reaction between two molecules of bilirubin monoglucuronide which is carried out by an enzyme located in the canalicular membrane of the liver cell. Based on these findings it is suggested that microsomal mono-conjugation of bilirubin may occur while it is bound to a glutathione-S-transferase and that the biliary excretion of bilirubin may involve interaction of a bilirubin monoglucuronide glutathione-S-transferase complex with the bile canalicular membrane with resultant excretion of the monoglucuronide or local formation and immediate excretion of the diglucuronide. It has been shown that both hepatic uptake and net hepatic clearance of conjugated bilirubin is substantially faster than that of the unconjugated pigment, presumably due, at least in part, to the ability of the former pigment to bypass the conjugation step. In addition, the hepatic uptake of conjugated bilirubin, like that of the unconjugated pigment, was inhibited by other organic anions, such as BSP and indocyanine green, but not by glycocholate, suggesting that uptake is not mediated by simple diffusion but by a more complex mechanism shared with unconjugated bilirubin.

To assess the potential role of membrane carriers in the hepatic transport of organic anions the kinetics of binding of labeled BSP to canalicular and plasma membrane fractions isolated from rat hepatocytes were studied. Saturable binding of BSP to both membranes was shown and this binding was competitively inhibited by bilirubin and indocyanine green but not by taurocholate. This investigation has provided further support for the hypothesis that organic anions other than bile salts share a common pathway for hepatic uptake and biliary excretion and that a membrane carrier protein may be involved in these processes.

Significance to Biomedical Research:

Studies of organic anion kinetics in patients with Gilbert's syndrome, Crigler-Najjar syndrome and normal volunteers have led to a marked improvement

in our understanding of the excretory function of the liver. In particular, these studies have demonstrated that it is possible to quantitate hepatic function in terms of clearances in a manner analagous to the use of creatinine clearance measurements for quantitating renal function. The ability to accurately measure both bilirubin turnover and bilirubin clearance, the two parameters which determine the plasma bilirubin concentration, has made possible a precise functional classification of all cases of unconjugated hyperbilirubinemia. Studies of the sort described above are also leading both to the development of new clinical methods for assessing the function of individual steps in organic anion transport processes and to an improved understanding of the nature of the defects in a variety of disease states.

Proposed Course:

We plan to continue our evaluation of organic anion transport in patients with Gilbert's syndrome. In particular, new approaches to studies of bilirubin kinetics involving the intravenous injection of various loading doses of bilirubin, in addition to tracer doses, will be employed in an attempt to see whether or not those patients with demonstrated abnormalities in the hepatic uptake of indocyanine green and sulfobromophthalein can also be shown to have a corresponding alteration in the hepatic uptake of bilirubin. In addition, the methods currently used for the studies of unconjugated bilirubin kinetics will be extended to permit studies of the fate of conjugated radiolabeled bilirubin. This will enable the application of these isotope techniques to a variety of more common disease states, including cirrhosis and viral hepatitis.

Publications:

1. Wolkoff, A. W., Scharschmidt, B. F., Plotz, P. H., and Berk, P. D.: Purification of conjugated bilirubin: A new approach utilizing albumin-agarose gel affinity chromatography. Proc. Soc. Exper. Biol. Med. 152: 20-23, 1976.

| SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space) | U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT | PROJECT NUMBER Z01 AM 53,501-04 DDB |
|---|---|---|

PERIOD COVERED
July 1, 1976 through September 30, 1977

TITLE OF PROJECT (80 characters or less)

Use of Artificial Support Devices for the Treatment of Fulminant Hepatic Failure

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

| | | | |
|---|---|---|---|
| P.I.: | P. D. Berk | Chief, Sec. on Dis. of the Liver | DDB:NIAMDD |
| Other: | B. L. Blitzer | Clinical Associate | DDB:NIAMDD |
| | E. A. Jones | Senior Investigator | DDB:NIAMDD |
| | H. Gralnick | Chief, Hematology Section | CP:CC |
| | I. J. Kopin | Chief, Lab. Clin. Science | LCS:NIMH |
| | P. F. Teychenne | Clinical Associate | ETB:NINCDS |
| | D. G. Goodman | Acting Chief, Tumor Pathology Sec. | EXP:NCI |

COOPERATING UNITS (if any)

None

LAB/BRANCH
Digestive Diseases Branch

SECTION
Section on Diseases of the Liver

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20014

| TOTAL MANYEARS: | PROFESSIONAL: | OTHER: |
|---|---|---|
| 2 | 1 1/2 | 1/2 |

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

An animal model of acute hepatic failure which closely resembles human fulminant hepatic failure has been developed in rabbits with a view to testing the effectiveness of hemoperfusion through albumin-agarose affinity chromatography columns as a means of providing temporary hepatic support. The model induced has the advantages of being (i) non-hazardous to personnel, (ii) fairly reproducible, (iii) potentially reversible and (iv) closely resembling human fulminant hepatic failure in its clinical, electroencephalographic, serum biochemical and hepatic histological features.

The hypothetical effects of different hemoperfusion schedules on the kinetics of depletion of a protein-bound substance unconjugated bilirubin have been simulated using a computer. The results suggest that simple alterations in the schedule of hemoperfusions might accelerate depletion of toxins from an extra-vascular pool and hence promote earlier arousal and possibly more frequent survival in patients with fulminant hepatic failure.

Project Description:

Objectives:

To develop artificial extracorporeal methods for removing from blood those metabolic products which are ordinarily extracted by the liver. The ultimate objective of this project is the development of an artificial support system for use in the management of acute potentially-reversible hepatic failure.

Methods Employed:

1) We have used the approach of affinity chromatography, using human serum albumin conjugated in high concentration to agarose beads as a prototype of a simple "artificial liver" for the removal of organic anions from the circulation. Bilirubin was selected as a test substance because of its extremely high binding to serum proteins, which makes it a particularly difficult substance to remove.

2) To generate an animal model of fulminant hepatic failure, the selective hepatotoxin galactosamine-HCl was administered intravenously according to a precise dose schedule to an inbred strain of rabbits.

Major Findings:

1) Affinity chromatography using albumin-agarose beads has been found to be a highly efficient means of removing bilirubin, BSP, indocyanine green and a number of physiologic metabolites from the circulation.

2) A hemoperfusion system has been designed which permits sequential perfusion of several albumin-agarose columns one at a time followed by sequential elution of removed substances from each column. The system enables continuous hemoperfusion while requiring only a small proportion of an animal's blood volume to be in the extracorporeal circuit.

3) This system has been shown to be effective in correcting both unconjugated and conjugated hyperbilirubinemia in rats and the neonatal hyperbilirubinemia of premature Rhesus monkeys.

4) The incorporation of citrate in perfusion fluids has been shown to be effective in minimizing loss of platelets and granulocytes from blood in the hemoperfusion system.

5) Galactosamine has been found to produce a highly specific hepatic necrosis the histology of which closely resembles that seen in some forms of fulminant hepatic failure in man. The biochemistry of this lesion has previously been well worked out by others and we have now established that a dosage regimen employing repeated injections in an inbred strain of rabbits produces death from hepatic failure in approximately 90% of the animals. Furthermore, death is preceded by a period of clinically evident coma of 4-6 hours duration. The model induced has the advantages of being (i) non-hazardous to personnel, (ii) fairly reproducible, (iii) potentially

reversible and (iv) closely resembling human fulminant hepatic failure in its clinical, electroencephalographic, serum biochemical and hepatic histologic features.

6) Arousal of patients from coma due to fulminant hepatic failure may be attributable, at least in part, to depletion of protein-bound substances toxic to the brain from a slowly exchanging extravascular pool such as the brain or CSF. The hypothetical effects of different hemoperfusion schedules on the kinetics of depletion of a protein-bound substance, unconjugated bilirubin, have been simulated using a computer. Data on the kinetics of unconjugated bilirubin in man and experimentally determined values for the efficiency of hemoperfusion through albumin-agarose columns in removing unconjugated bilirubin from plasma were used in the simulations. The results suggest that simple alterations in the schedule of hemoperfusions migh accelerate depletion of toxins from an extravascular pool and hence promote earlier arousal and possibly more frequent survival in patients with fulminant hepatic failure.

Proposed Course:

It is planned to determine the effects of various hemoperfusion schedules on various clinical, biochemical and electroencephalographic parameters of the galactosamine-induced animal model of hepatic coma. Initially hemoperfusions will be over albumin-agarose gel but subsequently other agents, such as activated charcoal and XAD-2 neutral resin, may be used.

Publications:


1. Berk, P. D., Scharschmidt, B. F., Martin, J. F., and Plotz, P. H.: Artificial support systems for liver failure. Kidney International 10: S233-S238, 1976.

2. Berk, P. D., Plotz, P. H., and Scharschmidt, B. F.: Removal of bilirubin from blood by affinity-competition chromatography over albumin-agarose gel. In Chang, T. M. S. (Ed.); Biomedical Applications of Immobilized Enzymes and Proteins, Vol. 1. New York, Plenum Press, 1976, pp. 297-317.

3. Scharschmidt, B. F., Martin, J. F., Shapiro, L. J., Plotz, P. H., and Berk, P. D.: The use of calcium chelating agents and prostaglandin $E_1$ to eliminate platelet and white blood cell losses resulting from hemoperfusion through uncoated charcoal, albumin-agarose gel, and neutral and cation exchange resins. J. Lab. Clin. Med. 89: 110-119, 1977.

4. Scharschmidt, B. F., Martin, J. F., Shapiro, L. J., Plotz, P. H., and Berk, P. D.: Hemoperfusion through albumin-conjugated agarose gel for the treatment of neonatal jaundice in premature Rhesus monkeys. J. Lab. Clin. Med. 89: 101-109, 1977.

| SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space) | U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT | PROJECT NUMBER Z01 AM 53,502-04 DDB |
|---|---|---|

PERIOD COVERED
July 1, 1976 through September 30, 1977

TITLE OF PROJECT (80 characters or less)
Clinical and Histologic Studies of Vinyl Chloride Associated Liver Disease

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

P.I.: P. D. Berk Chief, Sec. on Dis. of the Liver DDB:NIAMDD

COOPERATING UNITS (if any)
None

LAB/BRANCH
Digestive Diseases Branch

SECTION
Section on Diseases of the Liver

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20014

| TOTAL MANYEARS: | PROFESSIONAL: | OTHER: |
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| 0 | 0 | 0 |

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Terminated.

| SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space) | U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT | PROJECT NUMBER Z01 AM 53,503-03 DDB |
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PERIOD COVERED
July 1, 1976 through September 30, 1977

TITLE OF PROJECT (80 characters or less)

Immunologic Studies in Chronic Active Hepatitis and Primary Biliary Cirrhosis

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

| | | | |
|---|---|---|---|
| P.I.: | E. A. Jones | Senior Investigator | DDB:NIAMDD |
| Other: | P. D. Berk | Chief, Sec. on Dis. of the Liver | DDB:NIAMDD |
| | J. M. Vierling | Clinical Associate | DDB:NIAMDD |
| | C. J. Steer | Clinical Associate | DDB:NIAMDD |
| | S. P. James | Clinical Associate | DDB:NIAMDD |
| | W. Strober | Senior Investigator | MET:NCI |
| | D. L. Nelson | Investigator | MET:NCI |
| | M. M. Frank | Clin. Dir., NIAID | LCI:NIAID |
| | C. J. Jaffe | Clinical Associate | LCI:NIAID |

COOPERATING UNITS (if any)

None

LAB/BRANCH
Digestive Diseases Branch

SECTION
Section on Diseases of the Liver

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20014

| TOTAL MANYEARS: | PROFESSIONAL: | OTHER: |
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| 2 | 1 1/2 | 1/2 |

CHECK APPROPRIATE BOX(ES)

☒ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Abnormal immune mechanisms are being studied in patients with HB$_s$Ag negative chronic active hepatitis and primary biliary cirrhosis selected according to strict diagnostic criteria. A detailed immunologic evaluation is undertaken, which includes the following techniques: multiple intradermal antigen tests, primary immunization with both protein and polysaccharide antigens, quantitation of T and B cell populations using Rosette tests and immunofluorescence, HLA typing, measurement of serum immunoglobulins and functional assay of serum complement components. The in vitro cytotoxic capacity of lymphocytes and lymphocyte subpopulations (T, K and B cells) against several liver-specific and non-hepatic target cells in tissue culture is being evaluated. In addition, the capacity of the reticuloendothelial system to clear nonspecific and immunospecific particles from the circulation is being assessed. Nonspecific clearance is determined from the rate of clearance of $^{125}$I-microaggregated albumin and immunospecific clearance from the fate of $^{51}$Cr-labeled autologous red cells coated with a specific IgG or IgM antibody. The results of these studies include the demonstration of defects in spontaneous cell-mediated cytotoxicity and immunospecific reticuloendothelial clearance in primary biliary cirrhosis.

Project Description:

Objectives:

To evaluate in detail the immunologic status of patients with chronic active hepatitis and primary biliary cirrhosis with special emphasis on immunologic phenomena currently believed to be of pathogenetic significance in these diseases. The ultimate objective is to identify the major pathogenetic mechanisms in both diseases.

Methods Employed:

Criteria for patient selection include consistent history, physical examination, results of serum biochemical tests, serum non-organ specific antibody titers and hepatic histology. Patients with a history of ingestion of hepatotoxic drugs are excluded. To date the study has been be limited to $HB_sAg$ negative subjects. A few patients with alcoholic liver disease have been studied to generate control data in a chronic liver disease in which the etiology is not primarily immunologic. Similarly, a few patients with large duct biliary obstruction have been studied to generate control data in patients who have cholestasis not due to primary biliary cirrhosis.

The immunologic evaluation includes the following types of investigations: 1) Humoral and cellular immune responses are being assessed by multiple intradermal antigen tests; primary immunization with keyhole limpet hemocyanin and pneumococcal polysacchride with subsequent keyhole limpet hemocyanin skin testing. Quantitation of T and B cell populations using Rosette tests and immunofluorescence; HLA typing; quantitation of serum concentrations of immunoglobulins; and functional (hemolytic) assay of complement components are also included in the protocol. 2) The cytotoxic capacity of lymphocytes and lymphocyte subpopulations harvested from peripheral blood is assessed in an *in vitro* cytotoxicity assay, using target cells which include Chang human hepatoma cells and mouse EL-4 sarcoma cells. The assay depends on quantitating $^{51}Cr$ released from labeled target cells by lymphocytes from normal subjects and patients run in parallel. The technique is used to assay spontaneous cell-mediated cytotoxicity, mitogen induced cellular cytotoxicity (using phytohemagglutinin, concanavalin A and pokeweek mitogen) and antibody dependent cellular cytotoxicity (using a specific Chang cell antibody). 3) The capacity of the reticuloendothelial system to remove from the circulation nonspecific and immunospecific particles is being assessed. Nonspecific clearance is determined from the rate of clearance of a test dose of $^{125}I$-microaggregated albumin and hepatic blood flow from the rate of clearance of a tracer dose of this substance. Immunospecific clearance is investigated by following the fate of intravenously administered autologous red cells labeled with $^{51}Cr$ and coated with a pure IgM or IgG antibody directed against ABO or Rh antigens. Clearance of IgM coated cells occurs largely in the liver and depends on the interaction between C3b deposited *in vivo* on the red cell membrane and specific C3b receptors on Kupffer cells, while clearance of IgG coated cells depends on Fc-receptor-bearing fixed macrophages in the spleen. After clearance IgG-coated cells undergo phagocytosis, whereas IgM-coated cells either undergo phagocytosis or release back into the circulation as C3d-coated cells as a result of the action of circulating C3b inactivator.

Using this protocol it is possible to derive information about the function of both the afferent and efferent limbs of a patient's immune response. The assessment of the efferent limb includes data on humoral immunity (primary and secondary responses) and cellular immunity, including quantitation and functional assay of T, B and K cells.

Major Findings:

Results to date include the following:

1) There is a higher incidence of skin test anergy in patients with primary biliary cirrhosis than in patients with chronic active hepatitis and controls. This anergy includes the response to de novo immunization with keyhole limpet hemocyanin.

2) The cytotoxic response in patients with primary biliary cirrhosis and $HB_sAg$ negative chronic hepatitis is not specific for the Chang cell and does not indicate prior sensitization in vivo to antigens on this target cell.

3) In contrast to chronic hepatitis, spontaneous cell-mediated cytotoxicity (SCMC) of lymphocytes was shown to be defective in PBC. No defect in mitogen-induced cytotoxicity (MICC) or antibody-dependent cytotoxicity (ADCC) was demonstrated in either disease. In both diseases SCMC and ADCC were shown to be mediated by a subpopulation of lymphocytes which lack surface immunoglobulin and bear Fc receptors whereas MICC was mediated by lymphocytes which lack both surface immunoglobulin and Fc receptors. Thus, a difference in cytotoxic function in PBC and chronic hepatitis has been defined in that the former but not the latter disease is associated with a defect in the spontaneous cytotoxic function of lymphocytes which lack surface immunoglobulin but bear Fc receptors.

4) Starting with peripheral blood mononuclear cells it has been shown that depletion of complement receptor-bearing cells was associated with enrichment of Fc positive and surface immunoglobulin negative cells, depletion of phagocytes and surface immunoglobulin positive cells and normal or enhanced SCMC, MICC and ADCC. In contrast, depletion of Fc positive cells was associated with enrichment of complement receptor-bearing cells and virtually no capacity to induce SCMC or ADCC. These experiments indicate that at least the majority of cells which mediate SCMC, MICC and ADCC do not bear complement receptors.

5) The clearance of both tracer and test doses of microaggregated albumin was normal in all patients studied, indicating normal hepatic blood flow and a normal phagocytic capacity of the reticuloendothelial system in every subject studied. The clearance and subsequent phagocytosis of IgG-coated radiochromated autologous red cells was normal in all groups, except patients with PBC, the majority of whom had an increased rate of clearance. This observation may imply activation of the reticuloendothelial system in this disease. In contrast, the clearance of IgM-coated radiochromated autologous red cells was markedly decreased in each patient with PBC but was normal in all of the other subjects studied. To obtain further insight into this clearance defect in PBC, a compartmental model of the C3b clearance

process was developed. Using computer simulations the model was able to account for the observed data and showed that the major determinant of the defect was a reduced initial rate of removal of C3b-coated cells from the circulation. It was shown that the defect could not be attributed to a deficiency of components of the classical complement pathway, to increased or decreased activity of C3b-inactivator, or to competitive inhibition of C3b receptors on Kupffer cells by soluble immune complexes. However, the defect could be due to a decrease in the number or affinity of C3b-receptors or to partial saturation of these receptors by excess circulating C3b. That there is probably excess circulating C3b in PBC is suggested by the finding that there is hypercatabolism of C3 in this disease.

Significance to Biomedical Research:

Eludication of the pathogenetic mechanisms which initiate and sustain the disease process in chronic active hepatitis and primary biliary cirrhosis is required for better diagnostic testing and development of rational therapies. Defined initiating factors exist for those subgroups of chronic active hepatitis which are associated with $HB_sAg$ and which follow the ingestion of certain drugs, whereas initiating factors are unknown for the remainder of patients with chronic active hepatitis and all patients with primary biliary cirrhosis. The processes leading to sustained disease activity are unknown in both conditions. Current hypotheses favor immunologic mechanism(s). These hypotheses, in part, stem from the high prevalence of abnormal immunologic phenomena in both diseases. The empiric use of adrenal corticosteroids and other immunosuppressive therapies in these diseases appear to be, at least in part, a reflection of this view. To date, existing specific hypotheses lack experimental confirmation. In addition, it is not known whether certain immunologic abnormalities found in some subgroups of patients and not in others are epiphenomena or are related to pathogenesis.

The current study is designed to provide a comprehensive immunologic evaluation of selected patients so that the spectrum of immunologic abnormalities associated with primary biliary cirrhosis and $HB_sAg$ negative chronic hepatitis can be defined and the relationship of these abnormalities to clinical and histologic variables can be assessed. In addition, hypotheses of specific pathogenic mechanisms are tested. The significant morbidity and mortality of chronic active hepatitis, the uniform mortality of primary biliary cirrhosis, and the inadequacies of all current therapies in both conditions emphasize the need for information which throws light on the pathogenesis of these diseases.

Proposed Course:

The protocol, as described above, will be continued. Investigations will also be directed toward defining the antigenic specificity of the cell membrane of the Chang cell and other hepatic target cells, performing functional assays of lymphocyte subpopulations and developing methods for the isolation of pure preparations of hepatocytes from animal livers and from biopsies of human livers for use as target cells. If appropriate, T-cell suppressor function will be evaluated in collaboration with workers in the Immunophysiology Section of the Metabolism Branch, National Cancer Institute. After adequate

safeguards are instituted, patients with $HB_sAg$ positive chronic active hepatitis will be included within the protocol during the next year.

Publications:


1. Potter, B. J., Elias, E., and Jones, E. A.: Hypercatabolism of the third component of complement in patients with primary biliary cirrhosis. J. Lab. Clin. Med. 88: 427-439, 1976.

2. Berk, P. D., Jones, E. A., Plotz, P. H., Seeff, L. B., and Wright, E. C.: Corticosteroid therapy for chronic active hepatitis. Ann. Int. Med. 85: 523-525, 1976.

| SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space) | U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT | PROJECT NUMBER Z01 AM 53,504-03 DDB |
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PERIOD COVERED
July 1, 1976 through September 30, 1977

TITLE OF PROJECT (80 characters or less)
Studies of Copper Metabolism in Man Using $^{67}Cu$ and $^{64}Cu$

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

| | | | |
|---|---|---|---|
| P.I.: | E. A. Jones | Senior Investigator | DDB:NIAMDD |
| Other: | J. M. Vierling | Clinical Associate | DDB:NIAMDD |
| | R. Aamodt | Senior Radiobiologist | NM:CC |
| | W. Rumble | Biologist/Radiobiologist | NM:CC |
| | R. Shrager | Mathematician | LSMM:CR |

COOPERATING UNITS (if any)
None

LAB/BRANCH
Digestive Diseases Branch

SECTION
Section on Diseases of the Liver

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20014

| TOTAL MANYEARS: | PROFESSIONAL: | OTHER: |
|---|---|---|
| 1 1/2 | 1 | 1/2 |

CHECK APPROPRIATE BOX(ES)

[X] (a) HUMAN SUBJECTS [X] (b) HUMAN TISSUES [ ] (c) NEITHER

[ ] (a1) MINORS [ ] (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

To study copper metabolism in vivo the fate of $^{67}Cu$ is followed after its intravenous injection or the fate of both $^{67}Cu$ and $^{64}Cu$ are followed after giving one of the isotopes intravenously and the other by mouth. Subsequently, measurements are made of radioactivity in the whole body, liver, red cells, ceruloplasmin, ceruloplasmin-free plasma, urine and feces. The data obtained are being analyzed by computer using curve-fitting, deconvolution and integration techniques. The computer analysis enables the kinetics of intestinal absorption, hepatic uptake, biliary excretion and incorporation into ceruloplasmin to be quantitated. The primary goal of these studies is to obtain an improved understanding of copper metabolism in normal subjects and in patients with diseases associated with increased deposition of copper in the liver, notably Wilson's disease and primary biliary cirrhosis. Another goal is to determine whether a set of abnormalities of radiocopper kinetics can be defined which would enable subjects who are heterozygous for Wilson's disease to be distinguished from those who are homozygous and those who are neither homozygous nor heterozygous for this disease.



PHS-6040
(Rev. 10-76)

Project Description:

Objectives:

To evaluate quantitatively the metabolism of copper in health and specific diseases associated with abnormalities of copper metabolism.

The ultimate goals include a comprehensive understanding of normal copper metabolism, and an evaluation of the significance of specific abnormalities of copper metabolism in patients who have increased deposition of copper in the liver, in particular those with hepatolenticular degeneration (Wilson's disease) and with diseases associated with chronic cholestasis such as primary biliary cirrhosis. In addition, relatives of patients with hepatolenticular degeneration, some of whom are heterozygotes for this condition, are also studied, to determine whether abnormalities of copper metabolism characteristic of the heterozygote state can be defined.

Methods Employed:

1) Studies using $^{67}Cu$ alone. After the intravenous injection of $^{67}Cu$ serial measurements are made of whole body radioactivity (using a whole body counter), hepatic radioactivity (using a scintillation probe), and radioactivity in red cells, ceruloplasmin-free plasma, ceruloplasmin, urine and feces. With the exception of hepatic radioactivity, these measurements are made for a period of 13 days. Serum ceruloplasmin-bound $^{67}Cu$ determination involves the removal of non-ceruloplasmin-bound $^{67}Cu$ from serum samples by addition of a copper chelating agent and passage of samples down small columns of activated charcoal. Plasma nonceruloplasmin-bound $^{67}Cu$ is determined by subtracting radioactivity in ceruloplasmin from that in whole plasma. The curves of total body, hepatic, red cell, plasma ceruloplasmin and plasma nonceruloplasmin radioactivity are defined and the total excretion of radioactivity in urine and feces calculated.

2) Studies using $^{67}Cu$ and $^{64}Cu$. Studies are also being conducted in which the fate of simultaneously administered $^{67}Cu$ and $^{64}Cu$ is followed. One of the isotopes is given orally and the other intravenously. The subsequent protocol is the same as for studies using $^{67}Cu$ alone except that both $^{67}Cu$ and $^{64}Cu$ radioactivity are measured in all experimental specimens. In measuring $^{67}Cu$ and $^{64}Cu$ simultaneously an appropriate correction is made for the radioactivity due to $^{64}Cu$ which cannot be excluded when measuring $^{67}Cu$. The data obtained are being analyzed by computer. This enables various parameters of copper metabolism to be quantitated, in particular the kinetics of intestinal absorption, hepatic uptake, biliary excretion and incorporation into ceruloplasmin.

Major Findings:

1) The ranges of whole body $^{67}Cu$ retention for normals and homozygotes and heterozygotes for hepatolenticular degeneration have been defined.

2) Appreciable incorporation of $^{67}Cu$ into ceruloplasmin in normals and a similar degree of incorporation in heterozygotes for hepatolenticular

degeneration and in patients with primary biliary cirrhosis have been demonstrated. In contrast, incorporation of $^{67}Cu$ into ceruloplasmin in homozygotes for hepatolenticular degeneration was minimal. Thus, incorporation of radiocopper into ceruloplasmin can be used to differentiate between two copper overload states, Wilson's disease and primary biliary cirrhosis.

3) A pool of nonceruloplasmin-bound copper which rapidly exchanges with plasma nonceruloplasmin-bound copper exists in control subjects but is either saturated or absent in patients with PBC.

4) About 50% of orally administered radiocopper is absorbed in both normal subjects and patients with PBC.

5) In the presence of a positive copper balance in PBC, the degree of intestinal absorption and hepatic extraction of orally-administered radiocopper is inappropriate.

6) A major defect in copper metabolism in PBC is decreased biliary excretion of copper.

7) There is a decreased fractional rate of uptake of copper by the liver in Wilson's disease which is not attributable solely to an increased hepatic copper concentration.

8) Incorporation of $^{67}Cu$ into ceruloplasmin is almost linear for at least 7 days after its administration to both normal subjects and patients with hepatic copper overload suggesting rapid equilibration of $^{67}Cu$ with a large hepatic pool of copper which has a slow turnover rate and a loss pathway with a small fractional transfer rate to the precursor pool of copper for ceruloplasmin synthesis.

Significance to Biomedical Research

In certain disease states marked variations in the copper content of plasma and various organs are known to occur. However, the mechanisms by which copper homeostasis is maintained in health and disturbed in disease are poorly understood. Increasing attention has been focused on copper metabolism in hepatolenticular degeneration since the refutation of the concept that a low ceruloplasmin concentration is primarily responsible for manifestations of the disease. In hepatolenticular degeneration and in diseases which are associated with chronic cholestasis, such as primary biliary cirrhosis, chronic large duct biliary obstruction and progressive intrahepatic cholestasis of infancy and childhood, very high concentrations of copper have been demonstrated in the liver. It has been suggested that increased hepatic copper may contribute to tissue injury and that reduction of hepatic copper by copper chelation therapy may improve the prognosis in these diseases. By the use of the double radionuclide technique outlined it is possible to determine whether specific disorders characterized by increased hepatic deposition of copper are associated with particular defects in copper metabolism, such as abnormalities of intestinal absorption, hepatic uptake, biliary secretion and incorporation into ceruloplasmin. The pathogenesis of neither hepatolenticular degeneration nor primary biliary cirrhosis is known, and defects in hepatic

handling of copper in these diseases could be either directly related to their pathogenesis or secondary phenomena potentiating tissue injury.

By studying heterozygotes for hepatolenticular degeneration, the effect of the presence of a single gene for this condition on copper metabolism can be defined.

Proposed Course:

To achieve the stated objectives it is necessary to study more normal subjects, patients with hepatolenticular degeneration and their relatives, patients with primary biliary cirrhosis and other patients who may have abnormal copper metabolism.

The protocol, as defined above, will be continued. Emphasis is being placed on studying a sufficient number of volunteers to permit normal ranges for variables of copper metabolism to be established. In homozygotes for hepatolenticular degeneration and patients with primary biliary cirrhosis, analysis of the data will focus on the nature of abnormalities of copper metabolism in relation to the manifestations of the disease, while in heterozygotes for hepatolenticular degeneration analysis of the data will be largely directed towards defining defects in copper metabolism which can readily be measured and which enable the heterozygote state to be distinguished from normal subjects. Further investigations in man may involve direct analysis of copper excretion in bile using a multiple lumen enteric tube. Specific questions relating to radiocopper kinetics which cannot be answered by studies in humans will be investigated in rats or dogs using $^{67}Cu$ and $^{64}Cu$.

Publications:

1. Jones, E. A., Rabin, L., Buckley, C. H., Webster, G., and Owens, D.: Progressive intrahepatic cholestasis of infancy and childhood. Gastroenterology 71: 675-682, 1976.

| SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space) | U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT | PROJECT NUMBER Z01 AM 53,505-02 DDB |
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PERIOD COVERED
July 1, 1976 through September 30, 1977

TITLE OF PROJECT (80 characters or less)

Studies of the Metabolism of Radioiodinated Alpha-1-Antitrypsin and Desialylated Alpha-1-Antitrypsin in Man

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

| | | | |
|---|---|---|---|
| P.I.: | E. A. Jones | Senior Investigator | DDB:NIAMDD |
| Other: | P. D. Berk | Chief, Sec. on Dis. of the Liver | DDB:NIAMDD |
| | J. M. Vierling | Clinical Associate | DDB:NIAMDD |
| | C. J. Steer | Clinical Associate | DDB:NIAMDD |

COOPERATING UNITS (if any)

Nuclear Medicine Department, CC (Dr. P. Bradley-Moore)

LAB/BRANCH
Digestive Diseases Branch

SECTION
Section on Diseases of the Liver

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20014

| TOTAL MANYEARS: | PROFESSIONAL: | OTHER: |
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| 2 | 1 1/2 | 1/2 |

CHECK APPROPRIATE BOX(ES)

☒ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Highly purified biologically active alpha-1-antitrypsins (α1AT's) are being isolated from plasma using gel exclusion chromatography, Concanavalin A affinity chromatography, and DEAE ion exchange chromatography. Isolated preparations of α1AT, trace-labeled with radioiodine, are being used to obtain data on the turnover of α1AT in normal subjects, patients with α1AT deficiency and their relatives and patients with chronic hepatocellular disease. The protease inhibitor phenotypes of subjects donating plasma for isolation of α1AT and subjects undergoing α1AT turnover studies are determined. α1AT's, isolated from normal subjects (phenotype PiMM) and from patients with α1AT deficiency (phenotype PiZZ) have different degrees of sialylation. Each is labeled with a different isotope of iodine and the metabolism of the two labeled preparations are compared in the same individuals. In addition, the metabolism in vivo of α1AT which has been desialylated in vitro is being studied. The relationship between the degree of desialylation of α1AT and its rate of catabolism and the extent to which decreased hepatic release or increased hepatic uptake of α1AT contributes to the low plasma concentrations of α1AT in α1AT deficiency are being determined.

Project Description:

Objectives:

Alpha-1-antitrypsin (α1AT) is a plasma glycoprotein which contains sialic acid and is responsible for most of the trypsin inhibitory capacity of plasma. The objectives of this study are to obtain data on the metabolism of α1AT in normal subjects (phenotype PiMM), patients with α1AT deficiency (phenotype PiZZ), relatives of PiZZ individuals with one Z gene and patients with hepatocellular disease unassociated with α1AT deficiency. The possibility that the degree of sialylation of this protein, which is less in PiZZ than PiMM individuals, influences its metabolism in vivo will be investigated by comparing the metabolism of α1AT's isolated from PiMM and PiZZ individuals in the same subjects and by studying the metabolism of α1AT which has been desialylated in vitro.

Methods Employed:

1) The protease inhibitor genotypes of subjects donating plasma for isolation of α1AT and subjects undergoing studies of α1AT metabolism are determined using acid starch gel electrophoresis followed by crossed immunoelectrophoresis.

2) α1AT is isolated from plasma using gel exclusion chromatography, Concanavalin A affinity chromatography and DEAE ion exchange chromatography.

3) The purity of isolated α1AT is determined using gel electrophoresis, crossed immunoelectrophoresis, polyacrylamide gel electrophoresis and isoelectric focusing. Its biological activity is assessed by a kinetic assay of tryptic inhibitory capacity using an artificial substrate.

4) To quantitate the turnover of α1AT in man, pure biologically active α1AT trace labeled with radioiodine is administered intravenously and serial measurements of radioactivity in the whole body, plasma and urine are analyzed.

Major Findings:

1) Isolated (PiMM) α1AT has been shown to be immunochemically pure and to retain both its characteristic electrophoretic microheterogeneity on acid starch gel electrophoresis and its ability to inhibit trypsin.

2) The half life of radioiodinated human (PiMM) α1AT in the plasma of rabbits varies from 8.7 to 10.4 hours whereas that of the same protein after desialylation was less than 10 minutes.

3) Ranges for pool sizes, transfer rates between pools and synthetic and catabolic rates of α1AT in normal subjects have been defined.

4) In turnover studies of MM α1AT in normal (PiMM) volunteers, the serum concentration of α1AT was shown to vary independently of its fractional catabolic rate but correlated with the synthetic rate. Analysis of the

turnover data indicated that α1AT is catabolized in, or in close relationship to, the intravascular compartment.

5) In analogous studies in normal volunteers the desialylated protein was shown to be removed from plasma approximately 150 times faster than the intact protein. Its rapid disappearance from plasma was associated with an equally rapid uptake of the radioiodine label by the liver. This finding emphasizes that the catabolism of α1AT can be influenced by the molecular structure of the carbohydrate moiety and suggests the existence of hepatic receptors for desialylated glycoproteins in the human liver.

Significance to Biomedical Research:

Homozygous α1AT deficiency (phenotype PiZZ) may be associated with cholestasis in the neonatal period, cirrhosis, liver cell carcinoma and emphysema at a young age. In this condition, hepatocytes have been shown to contain globules of α1AT-like material which is devoid of sialic acid, and the α1AT in plasma, which is present in abnormally low concentration, has also been shown to have a reduced content of sialic acid. The relationship of the characteristic hepatic globules and the low plasma concentration of α1AT to the hepatic lesions associated with α1AT deficiency is unknown. This study is designed to investigate the metabolism of α1AT in α1AT deficiency. The relationship of the characteristic hepatic globules to the rate at which α1AT is delivered to plasma and to the concentration of α1AT in plasma will be assessed. In addition, the contribution of increased catabolism or hepatic uptake of α1AT to reduced plasma concentrations of α1AT will be determined. The study will establish the effect of variations in the molecular structure of α1AT (e.g. different degrees of sialylation) on its rate of endogenous catabolism or hepatic uptake. In particular, the hypothesis that there are hepatic receptors for exposed galactose residues on desialylated glycoproteins which facilitate the rapid hepatic clearance of these proteins will be tested in man. The effect of chronic hepatocellular disease on the liver's capacity to clear a desialylated glycoprotein will be determined. In addition, the influence of chronic hepatocellular disease per se on the metabolism of α1AT will be studied so that important control data can be generated for the evaluation of α1AT metabolism in patients with α1AT deficiency associated with chronic hepatocellular disease. The observation that desialylated glycoproteins appear to be metabolized by hepatic lysosomes suggests that measurements of hepatic radioactivity after administering a radioiodinated desialylated glycoprotein such as α1AT could be used as a test for hepatic lysosomal function.

Proposed Course:

It is proposed to administer labeled intact and desialylated ($Pi^{MM}$) α1AT to patients with α1AT deficiency (phenotype PiZZ) and to administer labeled α1AT isolated from patients with α1AT deficiency, who do not have associated hepatocellular disease, to both normal subjects and patients with α1AT deficiency. These studies will enable the contribution of decreased hepatic release of α1AT and increased hepatic uptake of α1AT to the low serum concentration of α1AT in α1AT deficiency to be assessed. Whether α1AT that is biosynthetically deficient in sialic acid behaves differently from that which is

deficient in sialic acid as a result of neuraminidase treatment will also be determined by these studies.

Publications: None

| SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space) | U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT | PROJECT NUMBER ZO1 AM 53,506-01 DDB |
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PERIOD COVERED
July 1, 1976 through September 30, 1977

TITLE OF PROJECT (80 characters or less)
Immunologic Properties of the Hepatic Asialoglycoprotein-Binding Protein

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

| | | | |
|---|---|---|---|
| P.I.: | E. A. Jones | Senior Investigator | DDB:NIAMDD |
| Other: | J. M. Vierling | Clinical Associate | DDB:NIAMDD |
| | C. J. Steer | Clinical Associate | DDB:NIAMDD |
| | J. W. Hickman | Research Chemist | LBM:NIAMDD |

COOPERATING UNITS (if any)
None

LAB/BRANCH
Digestive Diseases Branch

SECTION
Section on Diseases of the Liver

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20014

| TOTAL MANYEARS: | PROFESSIONAL: | OTHER: |
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CHECK APPROPRIATE BOX(ES)

☒ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

A glycoprotein isolated from rabbit hepatocytes (HBP) which has the ability to bind specifically desialylated glycoproteins is also capable of inducing mitogenesis in human desialylated lymphocytes. The immunogenic properties of HBP, the first lectin of mammalian origin to be recognized, is being studied further. It has been shown that isolated HBP has the ability to increase the cytotoxicity of human lymphocytes against Chang target cells to the same degree as the plant lectin phytohemagglutinin. HBP did not increase the cytotoxicity of intact lymphocytes. In addition, it has been shown that desialylated lymphocytes induced significantly more cytotoxicity in isolated rabbit hepatocytes, which bear HBP on their surface, than did intact lymphocytes, and that this phenomenon could be blocked by asialo-orosomucoid (a desialylated glycoprotein) but not by intact orosomucoid. These findings suggest that the cytotoxicity induced by membrane-bound HBP is similar in mechanism to that induced by the isolated protein and that the induced cytotoxicity probably depends on the interaction between HBP and exposed galactose residues in desialylated lymphocytes.

Project Description:

Objectives:

HBP is a normal constituent of the surface membranes of mammalian hepatocytes which can act as a mitogen. This project is designed to determine whether HBP has additional immunologic properties such as the ability to induce lymphocytes to become cytotoxic, that is to mediate mitogen-induced cellular cytotoxicity (MICC). The specificity of immunologic processes mediated by HBP, as well as the subpopulations of lymphocytes involved, will be assessed.

Methods Employed:

HBP is isolated in a pure form from rabbit liver using affinity chromatography. HBP in the surface membranes of hepatocytes is measured by its ability to bind $^{125}$I-asialo-orosomucoid. For assays of cytotoxicity lymphocytes are isolated from the blood of normal human volunteers by centrifugation over Ficoll-Hypaque. Lymphocytes are desialylated by incubating with neuraminidase. Two in vitro assays of cytotoxicity are used. One measures the release of radioactivity from $^{51}$Cr-labeled nucleated target cells after their incubation with effector cells in the presence or absence of mitogens. The other measures the loss of ability of target cells to adhere to plastic after incubation with effector cells. Viability of both effector and target cells is measured by the ability of cells to exclude the dye trypan blue. Chang cells are used as nonspecific nucleated target cells. Pure rabbit hepatocytes, isolated by perfusing the liver in situ with a solution containing collagenase and hyaluronidase, are used as hepatospecific target cells containing exposed HBP on their surface membranes.

Major Findings:

Cytotoxicity of desialylated lymphocytes against nonspecific nucleated target cells in the presence of HBP was equivalent to that in the presence of the powerful plant mitogen phytohemagglutinin and was much greater than that induced by these cells in the absence of HBP. After prolonged culturing of desialylated lymphocytes in the presence of HBP, this protein was found to induce blast transformation and the progeny cells exhibited increased cytotoxicity compared to desialylated lymphocytes cultured in the absence of HBP. Having shown that isolated rabbit hepatocytes contained exposed HBP in their surface membrane, it was found that a much greater proportion of these cells lost their ability to adhere to plastic after incubation with desialylated lymphocytes than after incubation with normal untreated lymphocytes. This effect of desialylated lymphocytes was specifically inhibited by asialo-orosomucoid, a desialylated glycoprotein, but not by intact orosomucoid. These findings indicate that HBP can mediate MICC not only as a purified protein but also as a constituent of the surface membrane of a mammalian hepatocyte.

Significance to Biomedical Research:

The importance of this study lies in the fact that for the first time

MICC has been shown to be mediated by a normal constituent of the surface membrane of a mammalian cell. The results demonstrate that a minor structural change in the surface membrane of lymphocytes might result in their becoming cytopathogenic when exposed to normal hepatocytes, suggesting that MICC may be a mechanism of hepatocellular injury. This observation raises the possibility that interaction between a variety of constituents of normal cell membranes and lymphocytes whose surface membranes have undergone subtle structural changes may be a mechanism of immune-mediated cellular injury in human disease.

Proposed Course:

The subpopulations of lymphocytes which mediate the immunologic effects of HBP will be determined.

It is planned to determine the mechanisms responsible for the known fate of desialylated lymphocytes in vivo. It has been demonstrated that intravenously administered neuraminidase-treated lymphocytes are preferentially sequestered in the liver of rats. In addition, it has been shown that incubation of rat thoracic duct lymphocytes with influenza virus at 37°C results in release of sialic acid as a consequence of the action of viral neuraminidase and that the desialylated lymphocytes so formed subsequently undergo preferential accumulation in the liver after their intravenous infusion. It seems that sequestration of desialylated lymphocytes in the liver is more likely to involve the binding of these cells to Kupffer cells than hepatocytes as circulating cellular elements during their passage through the normal liver are intimately exposed to Kupffer cells but not hepatocytes. Desialylation of lymphocytes exposes not only galactose residues which can react with HBP but also determinants to which IgM and IgG antibody molecules in plasma can react. Thus, the preferential localization of desialylated lymphocytes could occur as a consequence of HBP being present in the surface membrane of Kupffer cells or the immunospecific binding of these cells to Fc or C'3 receptors on Kupffer cells. Initial studies to clarify this issue will determine whether HBP can be demonstrated on the surface membrane of isolated Kupffer cells.

Publications: None

| SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space) | U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT** | PROJECT NUMBER ZO1 AM 54,003-05 DD |
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PERIOD COVERED
July 1, 1976 to September 30, 1977

TITLE OF PROJECT (80 characters or less)
Effect of Diabetic Control on Cholesterol Metabolism

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

| | | |
|---|---|---|
| PI: | L.J. Bennion, Staff Physician | PCRS, NIAMDD |
| Other: | P.H. Bennett, Acting Chief | PCRS, NIAMDD |

COOPERATING UNITS (if any)
S.M. Grundy, Chief of Metabolism VA Hospital, University of California, San Diego, California

LAB/BRANCH
Digestive Diseases Branch

SECTION
Phoenix Clinical Research Section

INSTITUTE AND LOCATION
NIAMDD, NIH, Phoenix, Arizona 85016

| TOTAL MANYEARS: | PROFESSIONAL: | OTHER: |
|---|---|---|
| 0.2 | 0.1 | 0.1 |

CHECK APPROPRIATE BOX(ES)

☒ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Six diabetic Pima Indians on no medications were kept in a hyperglycemic state at stable weight on a metabolic diet containing a known amount of cholesterol while measurements were made of blood and bile lipids and total body cholesterol balance. They were then treated with insulin and kept at the same weight while the same studies were repeated in the euglycemic state.



PHS-6040

Project Description:

Cholesterol balance bile acid pool size, bile acid composition, plasma lipids, and bile lipid composition were measured in six Pima Indians with maturity-onset diabetes mellitus and uncontrolled hyperglycemia (blood sugars over 250 mg/100ml).These measurements were then repeated after normoglycemia (blood sugars about 100 mg/100ml) had been achieved within insulin therapy.

Methods Employed: Cholesterol balance is determined by chemical methods using non-absorbable markers given by mouth. Fecal steroids are quantitated by gas-liquid-chromatography. Isotope dilution methods are employed in the determination of bile acid pool size. Bile lipid composition is determined by standard chemical procedures on bile obtained by duodenal siphonage.

Major Findings: Cholesterol balance (13.5 versus 11.0 mg/kg/day, $p<.05$), fecal bile acid excretion (415 versus 261 mg/day, $p<.05$), bile acid pool size (3150 versus 1950 mg, $p<.05$), fasting plasma cholesterol (193 versus 160 mg%, $p<.05$) and plasma triglycerides (251 versus 150 mg%, $p<.05$) were higher during uncontrolled hyperglycemia than during relative euglycemia on insulin. Gallbladder bile was significantly more saturated with cholesterol (181% versus 114%, $p<.05$) during insulin treatment than during hyperglycemia.

Significance to Biomedical Research and the Program of the Institute: The findings suggest that cholesterol synthesis is increased in the diabetic hyperglycemic state. If this conclusion is confirmed, it may provide an explanation for the increase in atherosclerosis among diabetics.

Proposed Course: This investigation has been completed.

Publications:

Bennion, L.J., and Grundy, S.M.: Effects of diabetes mellitus on cholesterol metabolism in man. N. Engl. J. Med. 296:1356-1371, 1977.

| SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space) | U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT** | PROJECT NUMBER Z01 AM 54,007-04 DD |
|---|---|---|

PERIOD COVERED
July 1, 1976 to September 30, 1977

TITLE OF PROJECT (80 characters or less)

Effects of short-term fasting on biliary lipid metabolism

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

| | | |
|---|---|---|
| PI: | L.J. Bennion, Staff Physician | PCRS, NIAMDD |
| Other: | P.H. Bennett, Acting Chief | PCRS, NIAMDD |

COOPERATING UNITS (if any)
None

LAB/BRANCH
Digestive Diseases Branch

SECTION
Phoenix Clinical Research Section

INSTITUTE AND LOCATION
NIAMDD, NIH, Phoenix, Arizona 85016

| TOTAL MANYEARS: | PROFESSIONAL: | OTHER: |
|---|---|---|
| 0.4 | 0.2 | 0.2 |

CHECK APPROPRIATE BOX(ES)

☒ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Gallbladder bile lipid composition, bile acid composition, bile acid pool size and turnover rates were measured in 6 normal humans before and during a 4-6 day fast. One subject admitted for this project was found to have endometrial carcinoma and had biliary lipid studies before and after bilateral oophorectomy.

Project Description:

Isotope dilution techniques were used to measure bile acid turnover and pool size in the fed state and again after a four-six day fast in six subjects. In addition, the lipid composition of gallbladder bile was examined in these subjects during the fed and the fasted state.

Methods Employed: Isotopic cholic acid was administered orally and gallbladder bile obtained on the four subsequent mornings. After the fourth bile sample, each subject fasted for five-six days. On the evening of the fourth fasting day, cholic acid labelled with a different isotope was administered and gallbladder bile obtained the two subsequent mornings. Total bile acid pool size and cholic acid pool size were calculated from the specific activity in bile obtained the first control day and again the first fasting day. This method of pool size determination has been validated previously in this laboratory and is precise to $\pm$2.6%. Cholic acid synthesis and fractional catabolic rates were calculated by standard methods for the fed state. Calculation of these parameters for the fasting state was achieved by assuming a single pool model for cholic acid and rapid changes in synthesis and fractional catabolic rates at the beginning of the fast. Gallbladder bile lipid composition and individual bile acid composition were measured by standard techniques.

Major Findings: Total bile acid pool size and cholic acid pool were either slightly reduced or unchanged by fasting. Cholic acid synthesis was reduced by a factor of three or four in all subjects during fasting. The fractional catabolic rate of cholic acid was reduced by fasting in all subjects. Fasting reduced the molar percent of cholesterol (relative to the solubilizing lipids, bile acids and lecithin) in all but one subject. Individual bile acid composition did not show any consistent change during fasting.

Significance to Biomedical Research and the Program of the Institute. These findings show that regulation of bile acid synthesis in man is influenced by feeding and fasting. The finding that bile cholesterol saturation is reduced by a 4-6 day fast was unexpected; it allays fears (based on known diurnal variations in bile composition) that fasting might increase the risk of cholesterol gallstone formation.

Proposed Course: Investigative work has been completed. Project has been discontinued.

Publications:

Duane, W.C., Ginsberg, R.L., and Bennion, L.J.: Effects of fasting on Bile Acid Metabolism and Biliary Lipid Composition in Man. J. Lipid Res. 17:211-219, 1976.

Duane, W.C.: Taurocholate- and taurochenodeoxycholate-lecithin micelles: the equilibrium of bile salt between aqueous phase and micelle. Biochem. Biophys. Res. Comm. 74:223-229, 1977.

Publications, cont.

Bennion, L.J.: Changes in Bile Lipids Accompanying Oophorectomy in a Premenopausal Woman. N. Engl. J. Med. (in press)

| SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space) | U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT** | PROJECT NUMBER ZO1 AM 54,009-04 DD |
|---|---|---|

PERIOD COVERED

July 1, 1976 to September 30, 1977

TITLE OF PROJECT (80 characters or less)

Effect of Phenobarbital Upon Biliary Lipid Metabolism

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: P.H. Bennett, Acting Chief PCRS, NIAMDD

Other: None

COOPERATING UNITS (if any)

None

LAB/BRANCH

Digestive Diseases Branch

SECTION

Phoenix Clinical Research Section

INSTITUTE AND LOCATION

NIAMDD, NIH, Phoenix, Arizona 85016

| TOTAL MANYEARS: | PROFESSIONAL: | OTHER: |
|---|---|---|
| 0.1 | 0.1 | 0 |

CHECK APPROPRIATE BOX(ES)

☒ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Recent investigations have indicated a possible beneficial effect of phenobarbital alone or in conjunction with chenodeoxycholic acid in the treatment of cholesterol gallstones. Hepatic cholesterol secretion, hepatic phospholipid secretion, bile acid secretion, gallbladder bile lipid composition, and bile acid composition, bile acid turnover and pool size have been measured in normal human volunteers before and after chronic administration of phenobarbital.



PHS-6040

Project Description:

Recent studies in primates have shown that phenobarbital, a known potent inducer of liver microsomal enzymes, had the following effects on biliary lipid metabolism and composition: 1) increased bile flow rates; 2) increased bile salt and phospholipid secretion with a concomitant decrease in cholesterol secretion by the liver; 3) a consequent decreased cholesterol content in bile relative to bile salts and phospholipids; 4) augmented bile salt synthesis rate and bile salt pool size in animals with intact enterohepatic circulations; 5) enhancement of the maximal bile salt secretion rate in animals with total biliary fistulae.

Studies in human subjects with cholesterol gallstones have shown that phenobarbital may decrease bile cholesterol saturation and have a useful synergistic role with chenodeoxycholic acid in the dissolution of cholesterol gallstones.

Methods Employed: Normal vaolunteers were given a tracer dose of radioactively labelled cholic acid, and total bile acid pool size, catabolic rate of cholic acid, and synthesis of cholic acid were determined from the fall in specific activity of cholic acid over the next four days. Hourly secretion rates of cholesterol, bile salts, and phospholipids were measured by the previously described method of Grundy and Metzger. All studies were performed on the same patients before and after the ingestion of phenobarbital for two months at a dose of 3 mg/Kg per day; each patient served as his own control. In addition, several control subjects were studied in the same manner except they took a placebo instead of phenobarbital.

Major Findings: Phenobarbital did not significantly alter any of the parameters of biliary lipid metabolism which we measured.

Significance to Biomedical Research and the Program of the Institute: Phenobarbital, at least when used alone, does not appear to be a useful agent for prevention or dissolution of cholesterol gallstones.

Proposed Course: This investigation has been completed. The project has been terminated.

Publications:

Ginsberg, R.L., and Garnick, M.B.: The Effect of Phenobarbital on Biliary Lipid Metabolism in Normal Man. Gastroenterology 72:1221-1227, 19[illegible].

| SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space) | U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT** | PROJECT NUMBER ZO1 AM 54,010-04 DD |
|---|---|---|

PERIOD COVERED
July 1, 1976 to September 30, 1977

TITLE OF PROJECT (80 characters or less)
Hepatic 3-Hydroxy-3-methylglutaryl (HMG) CoA Reductase Activity in Hamsters on a Lithogenic Diet

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT


PI: P.H. Bennett, Acting Chief — PCRS, NIAMDD
E.V. Flock, Visiting Scientist — PCRS, NIAMDD


COOPERATING UNITS (if any)
Center for Disease Control, Phoenix Laboratories

LAB/BRANCH
Digestive Diseases Branch

SECTION
Phoenix Clinical Research Section

INSTITUTE AND LOCATION
NIAMDD, NIH, Phoenix, Arizona 85016

| TOTAL MANYEARS: | PROFESSIONAL: | OTHER: |
|---|---|---|
| 0.1 | 0.1 | 0.0 |

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES XX ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)


The purpose of this investigation was to determine the effect of a lithogenic diet (one which is known to cause gallstones in hamsters) on the activity of HMG CoA reductase, the rate limiting enzyme in the synthesis of cholesterol. Syrian hamsters were either fed a normal diet or a lithogenic diet and then at various periods were killed. The activity of hepatic HMG CoA reductase was determined. Increases in the activity of HMG CoA reductase were found early in hamsters on the lithogenic diet and cholesterol gallstones developed later. The elevation of this enzyme is indicative of increased synthesis as a responsible factor in the genesis of cholesterol gallstones in these hamsters.

Project Description:

The development of cholesterol gallstones in young hamsters was investigated by feeding these animals a diet known to induce gallstones.

Objectives: The purpose of this investigation was to measure the rate-limiting enzyme for the synthesis of cholesterol, HMG CoA reductase. In addition, cholesterol concentrations were measured in carcass, liver and hepatic microsomes. The bile acid composition in gallbladder bile was determined.

Methods Employed: Hamsters were fed either a lithogenic diet or a normal chow diet. After varying intervals of time, the animals were killed and the livers and gallbladders removed. The cholesterol content and the activity of HMG CoA reductase were determined from the liver samples. The gallbladders were examined for the presence of cholesterol gallstones and the biliary composition of bile acids was determined.

Major Findings: None of the chow-fed hamsters developed gallstones, but 74% of those on the lithogenic diet for 26 to 49 days had gallstones. As early as 2 to 15 days on the lithogenic diet the mean hepatic HMG CoA reductase activity at 4 to 6 hours after the onset of the dark period was 20 times greater than in hamsters in the fasting state and 12 times that of fed hamsters on the chow diet. Similar elevations of enzyme activity were found in hamsters on the lithogenic diet for 26 to 49 days when gallstones had developed. The greatly stimulated enzyme activity could account for the increased secretion of cholesterol in bile of hamsters on the lithogenic diet noted by others with subsequent super-saturation of gallbladder bile with respect to cholesterol, and then gallstone formation.

The percentage of the bile acid pool represented by chenodeoxycholic acid was significantly higher, and that of cholic acid correspondingly lower on the gallstone-inducing diet. This shift in bile acid composition may have contributed to gallstone formation.

Proposed Course: This investigation has been completed. The results have been published. The project has been discontinued.

Publication:

Ginsberg, Ronald L., Duane, William C., and Flock, Eunice V.,: Hepatic 3-Hydroxy-3-Methylglutaryyl CoA Reductase Activity in Hamsters on a Lithogenic Diet. J. Lab. Clin. Med. 89:928-936, 1977.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)

U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE
PUBLIC HEALTH SERVICE
**NOTICE OF INTRAMURAL RESEARCH PROJECT**

PROJECT NUMBER

Z01 AM 54,012-03 DD

PERIOD COVERED

July 1, 1976 to September 30, 1977

TITLE OF PROJECT (80 characters or less)

Effect of Dietary Fiber Upon Biliary Lipid Composition

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: P.H. Bennett, Acting Chief PCRS, NIAMDD

Other: None

COOPERATING UNITS (if any)

M. Hendrikx, Research Dietician, PCRS, IHS

M. Garnick, Sidney Farber Cancer Institute, Harvard Med. School, Boston,Mass.

LAB/BRANCH

Digestive Diseases Branch

SECTION

Phoenix Clinical Research Section

INSTITUTE AND LOCATION

NIAMDD, NIH, Phoenix, Arizona 85016

| TOTAL MANYEARS: | PROFESSIONAL: | OTHER: |
|---|---|---|
| 0.1 | 0 | 0.1 |

CHECK APPROPRIATE BOX(ES)

☒ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Populations ingesting a high fiber content in their diet are reputed to have a lower incidence of cholesterol cholelithiasis. Consequently, we measured gallbladder bile lipid composition and bile acid composition in 5 normal humans after ingestion of a low bran diet and again after ingestion of an otherwise identical high bran diet to determine the effects of dietary fiber on bile composition.



PHS-6040

Project Description:

Objectives: Recent observations have suggested an association of cholesterol gallstones and diets high in refined carbohydrate content and low in bulk constituents. Diets high in fiber have also been associated with large total bile acid pool sizes in man as well as an increased proportion of chenodeoxycholic acid in bile. The purpose of the present study was to investigate the effect of a high bran (fiber) diet on the cholesterol content of gallbladder bile.

Methods Employed: Gallbladder bile was collected from normal humans before and after a 4-8 week period of high fiber (bran) consumption. The bile was analyzed for the relative proportions of cholesterol, bile acid, and phospholipid content as well as the proportions of individual bile acid species comprising the total bile acid pool.

Major Findings: No significant changes in bile composition resulted from ingestion of a high bran diet for 4-8 weeks.

Significance to Biomedical Research and the Program of the Institute. It is unlikely that the lower prevalence of cholelithiasis in populations eating large amounts of fiber is attributable to the fiber content of the diet. Increasing dietary fiber intake does not appear useful for the prevention of cholelithiasis.

Proposed Course: The investigation has been completed. The findings are being published and the project is terminated.

Publications:

Garnick, M.B., Ginsberg, R.L., Hendrikx, M.E., and Bennett, P.H.: Dietary fiber and biliary lipid composition. Amer. J. Clin. Nutrition (in press)

| SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space) | U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT** | PROJECT NUMBER Z01 AM 54,013-03 DD |
|---|---|---|

PERIOD COVERED
July 1, 1976 to September 30, 1977

TITLE OF PROJECT (80 characters or less)

Effect of Oral Contraceptives on Glucose Tolerance

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT


PI: P.H. Bennett, Acting Chief PCRS, NIAMDD
Other: P.J. Savage, Staff Physician SFSS, NIAMDD


COOPERATING UNITS (if any)
S.L. Aronoff, Dept. of Medicine, Washington University, St. Louis, MO

LAB/BRANCH
Digestive Diseases Branch

SECTION
Phoenix Clinical Research Section

INSTITUTE AND LOCATION
NIAMDD, NIH, Phoenix, Arizona 85016

| TOTAL MANYEARS: | PROFESSIONAL: | OTHER: |
|---|---|---|
| 0.1 | 0.1 | 0.1 |

CHECK APPROPRIATE BOX(ES)

☒ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)


Oral contraceptives were previously shown to induce an abnormal cortisone glucose tolerance in subjects who had otherwise normal oral glucose tolerance tests. To investigate the mechanisms of this change, subjects planning to start or stop oral contraceptives were tested by means of an oral glucose tolerance test and cortisone glucose tolerance test with the measurement of glucose and insulin responses during each test, both before, during and after taking oral contraceptives.




PHS-6040
(Rev. 10-76)

Project Description:

Objectives: The effect of oral contraceptives on carbohydrate metabolism has stimulated much interest, but in spite of the substantial amount of research the results have been, in some instances, contradictory.

The study was designed to verify and further investigate our observation of an abnormal cortisone glucose tolerance test in subjects on combined oral contraceptives in the presence of normal oral glucose tolerance test.

Methods Employed: Female volunteers (with a negative family history for diabetes) who were planning to start or to stop oral contraceptives were studied. An oral glucose tolerance test and a cortisone glucose tolerance test were performed on each woman while on oral contraceptives and again after stopping them or vice versa.

Major Findings: Although the oral glucose tolerance test was similar in each woman on or off oral contraceptives, plasma glucose levels during the cortisone glucose tolerance test were consistently higher while on oral contraceptives. Serum insulin levels were measured during each of the testing periods.

Significance to Biomedical Research and the Program of the Institute. This study should delineate the effects of oral contraceptives on glucose and insulin response during the oral glucose tolerance test and the cortisone glucose tolerance tests.

Proposed Course: Ten women have been completely tested. The data are being prepared for publication.

Publications: None

| SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space) | U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT** | PROJECT NUMBER ZO1 AM 54,014-03 DD |
|---|---|---|

PERIOD COVERED
July 1, 1976 to September 30, 1977

TITLE OF PROJECT (80 characters or less)
Low Density Lipoprotein Metabolism in American Indians

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: P.H. Bennett, Acting Chief PCRS, NIAMDD
Other: None

COOPERATING UNITS (if any)
M. Garnick, Sidney Farber Cancer Institute, Harvard Med. School, Boston, Mass.
T. Langer, Dept. of Medicine, Univ. of Pennsylvania

LAB/BRANCH
Digestive Diseases Branch

SECTION
Phoenix Clinical Research Section

INSTITUTE AND LOCATION
NIAMDD, NIH, Phoenix, Arizona 85016

| TOTAL MANYEARS: | PROFESSIONAL: | OTHER: |
|---|---|---|
| 0.1 | 0 | 0.1 |

CHECK APPROPRIATE BOX(ES)

☒ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Southwestern American Indians have lower serum cholesterol levels and lower prevalence of atherosclerosis than Caucasian control groups. To characterize these differences, we studied the lipoprotein cholesterol content and the kinetics of radiolabelled autologous low density lipoprotein in Southwestern American Indians and controls.



PHS-6040

Project Description:

Objectives: The low serum cholesterol levels and low prevalence of vascular disease (ASCVD) in southwestern American Indians compared to non-Indian populations, as well as the abnormalities in LDL kinetics observed in subjects with Type II hypercholesterolemia, provoked us to study LDL kinetics in American Indians.

Method Employed:

1. Patients were stabilized on a low cholesterol, low saturated fat diet for three weeks.
2. LDL was isolated from plasma by ultracentrifugation.
3. LDL was iodinated with $^{125}I$ by the iodine monochloride method.
4. The preparation was tested for immunologic purity (agarose gel electrophoresis), pyrogens and sterility according to U.S.P. standards.
5. The radio-labelled preparation was injected intravenously; plasma decay curves and urinary excretion of radioactivity were measured daily to determine the T 1/2 and FCR, LDL synthetic rate.

Major Findings: The absolute synthetic rate and total pool of apo-LDL were significantly lower in the Indians than Caucasians. In addition, concentrations apo-LDL and LDL cholesterol were significantly lower and the concentration of HDL cholesterol was higher in Indians than Caucasians.

Significance to Biomedical Research and the Program of the Institute: These findings help explain the low prevalence of coronary artery disease among southwestern American Indians, and reinforce the current notion that low levels of LDL and high levels of HDL protect against ASCVD. They also elucidate the mechanisms responsible for the low levels of LDL cholesterol observed among Southwestern American Indians.

Proposed Course: This study has been completed and the results are being prepared for publication.

Publication: None

| SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space) | U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT** | PROJECT NUMBER Z01 AM 54, 015-03 DD |
|---|---|---|

PERIOD COVERED
July 1, 1976 to September 30, 1977

TITLE OF PROJECT (80 characters or less)

Age of Onset of Lithogenic Bile in Southwest American Indians

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT


| | | |
|---|---|---|
| PI: | L.J. Bennion, Staff Physician | PCRS, NIAMDD |
| Other: | P.H. Bennett, Acting Chief | PCRS, NIAMDD |
| | A. Spagnola, Staff Associate | SFSS, EFSB, NIAMDD |
| | D. Mott, Staff Fellow | PCRS, NIAMDD |


COOPERATING UNITS (if any)

P.H. Klein, Argonne National Laboratory

LAB/BRANCH
Digestive Diseases Branch

SECTION
Phoenix Clinical Research Section

INSTITUTE AND LOCATION
NIAMDD, NIH, Phoenix, Arizona 85016

| TOTAL MANYEARS: | PROFESSIONAL: | OTHER: |
|---|---|---|
| 1.2 | 0.9 | 0.3 |

CHECK APPROPRIATE BOX(ES)

☒ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☐ (c) NEITHER

☒ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)


Lipid composition of gallbladder bile, bile acid pool size and sex hormone levels are being determined in Pima Indians below 21 years of age to determine the age at which Pima Indian bile becomes lithogenic These data will indicate the approximate duration of bile lithogenicity prior to the appearance of cholesterol gallstones and indicate the period of life at which prophylactic measures against gallstone formation may be taken. Bile is obtained by duodenal siphonage after stimulation of gallbladder contraction and bile acid pool size is measured by isotope dilution using deuterated chenodeoxycholic acid. Changes in bile lipids will be correlated with age, sex, level of sex hormones, pubertal development, body size, and plasma lipoprotein patterns.




PHS-6040
(Rev. 10-76)

Project Description:

Objectives: The lipid composition of gallbladder bile, bile acid pool size and sex hormone levels will be determined in Pima Indians of each sex between 9 and 22 years of age.

Methods Employed: Bile is obtained by duodenal siphonage after stimulation of gallbladder contraction by intraduodenal infusion of amino acids. Chenodeoxycholic acid pool size is measured by the mass ratio of deuterated chenodeoxycholic acid to native chenodeoxycholic acid in the bile twelve hours after the intragastric administration of dideutero-chenodeoxycholic acid. Determination of bile acid composition by gas-liquid chromatography will permit calculation of total bile acid pool size from the chenodeoxycholic acid pool size determined as above by mass-spectrometry. Bile lipid composition will be determined by standard chemical methods and expressed as percent saturation with cholesterol. Correlations of bile composition and bile acid pool size, blood levels of sex hormones, age, pubertal development, body size, sex, and plasma lipoproteins will be evaluated by multiple regression analysis.

Major Findings: Bile saturation increases between age 10 and 21.

Significance to Biomedical Research and the Program of the Institute: These studies should disclose whether the secretion of abnormally saturated bile is acquired in the pubertal or teen-age period among Pima Indians, or whether it is present in childhood. They should also disclose the relationship between the onset of lithogenic bile and the prevalence of cholelithiasis, since the prevalence of the latter has been carefully determined in this population. They should also elucidate the role of the sex hormones in the production of lithogenic bile, as well as the importance of bile acid pool size in the production of lithogenic bile in this population. Comparison of body size measurements and of age to bile acid pool size may yield important data regarding the regulation of bile acid pool size.

Proposed Course: The samples have been collected and are being analyzed.

Publication: None

| SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space) | U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT** | PROJECT NUMBER Z01 AM 54,016-02 DD |
|---|---|---|

PERIOD COVERED

July 1, 1976 to September 30, 1977

TITLE OF PROJECT (80 characters or less)

High Density Lipoprotein Cholesterol in Indians and Caucasians

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

| | | |
|---|---|---|
| PI: | P.H. Bennett, Acting Chief | PCRS, NIAMDD |
| Other: | B.V. Howard, Staff Fellow | PCRS, NIAMDD |
| | L.J. Bennion, Staff Physician | PCRS, NIAMDD |
| | P.J. Savage, Staff Physician | SFSS, NIAMDD |

COOPERATING UNITS (if any)

None

LAB/BRANCH

Digestive Diseases Branch

SECTION

Phoenix Clinical Research Section

INSTITUTE AND LOCATION

NIAMDD, NIH, Phoenix, Arizona 85016

| TOTAL MANYEARS: | PROFESSIONAL: | OTHER: |
|---|---|---|
| 0.3 | 0.2 | 0.1 |

CHECK APPROPRIATE BOX(ES)

☒ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Recent studies have implicated the importance of high density lipoprotein cholesterol as being an independent risk factor in the genesis of atherosclerosis. Studies have shown that individuals with a low amount of high density lipoprotein cholesterol tend to be at more risk for the development of ischemic heart disease and myocardial infarction. We are studying lipoprotein composition in Indians and Caucasians. Cholesterol and triglycerides are being measured in high density lipoproteins as well as other lipoprotein subclasses isolated by ultracentrifugation. These parameters are being compared in groups of normal and diabetic Indians with and without coronary artery disease. From this study we will attempt to determine if diabetes is associated with changes in serum lipoproteins, and to delineate the importance of high density lipoprotein cholesterol values in the pathogenesis of ischemic heart disease.



PHS-6040

Project Description:

Objectives:

1. Studies of the relationship between HDL cholesterol and ischemic heart disease. Recent studies have suggested there may be a relationship between HDL cholesterol levels and atherosclerotic heart disease. Our objective is to quantify HDL levels in a series of normal and diabetic Indians with and without coronary artery disease. Study of HDL in this defined population should yield information on the role of this lipoprotein in the etiology of atherosclerosis.

2. Determination of lipoprotein composition in normal and diabetic Indians. Previous studies have yielded data on total serum cholesterol and triglyceride levels in the Pima Indian population. In this study lipoprotein classes will be isolated by ultracentrifugation and cholesterol and triglyceride quantitated in the isolated lipoproteins. These parameters will be compared in normal and diabetic individuals, to determine if diabetes is associated with changes in lipoprotein composition.

Major Findings:

1. Studies on the relationship between HDL cholesterol and ischemic heart disease.

The heparin-manganese chloride method for isolating HDL has been investigated for use with frozen plasma samples. HDL cholesterol was measured in samples from 7 individuals before and after 2-9 months of freezing. No significant differences in HDL cholesterol values were observed ($44.3 \pm 4.4$ mg/dl vs. $42.1 \pm 5.4$ mg/dl.) This method will allow us to assay previously frozen samples from selected individuals.

2. Determination of lipoprotein composition in normal and diabetic Indians.

Studies are currently in progress to isolate lipoproteins and quantitate cholesterol and triglycerides in fasting plasma samples from normal and diabetic Pima Indians admitted to the clinical research unit and placed on a standard diet. Results have been analyzed for a selected group of 25 females from 20 to 35 years of age. The data to date (table 1) indicate that in diabetics there appears to be a significant decrease in HDL cholesterol, and a decrease in the ratio of HDL to LDL cholesterol. In addition, the diabetics have higher total and VLDL triglycerides.

TABLE I ZO1 AM 54,016-02 DD

Lipoprotein Cholesterol and Triglycerides in Normal and Diabetic Pima Indians

| Cholesterol levels (mg/100 ml) | Normal | Diabetic |
|---|---|---|
| total | 189±12 | 193±13 |
| VLDL | 15±2.4 | 14±38 |
| LDL | 114±9.3 | 124±10 |
| HDL | 52±4.2 | 43±1.6 |
| Triglyceride levels (mg/100 ml) | | |
| total | 138±22 | 184±10 |
| VLDL | 81±19 | 113±12 |
| LDL | 20±3.6 | 38±3.7 |
| HDL | 7.0±1.07 | 13±1.6 |
| LDL Cholesterol/protein | 1.3±0.07 | 1.4±0.10 |
| HDL cholesterol/LDL cholesterol | 0.48±0.04 | 0.38±0.03 |
| Number of subjects | 11 | 14 |
| Age (years) | 27 | 26 |
| height (cms) | 159 | 161 |
| weight (Kg) | 92 | 106 |

Free fatty acid levels are also being measured in fasting samples from normal and diabetic Pima Indians in order to determine if levels of this rapidly turning over plasma lipid are reflective of alterations in lipid metabolism or correlated with insulin levels in this population. Analysis of the data in a group of 20 females in the age range 20-35 years indicates fasting values of 13.8±1.6 mg/dl in the normals and 17.3±1.2 mg/dl in the diabetics.

Significance to Biomedical Research and the Program of the Institute:

This study will determine whether changes in lipoprotein composition, and especially HDL cholesterol levels, are associated with diabetes and

the development of ischemic heart disease. The data obtained on HDL and other lipid classes will thus help elucidate the relationship of these lipoproteins to the development of atherosclerosis.

In addition, this study should yield information on lipoprotein composition in groups of normal and diabetic Pima Indians. Since this population serves as a carefully defined model for adult onset diabetes, the data should contribute to an understanding of the effects of diabetes on lipoprotein metabolism and the relationship of diabetes to atherosclerosis.

Proposed Course:

1. Studies of the relationship between HDL cholesterol and ischemic heart disease.

   A group of normal and diabetic Pima Indians have been identified who have evidence of ischemic heart disease as determined by abnormal EKG's. Frozen samples of plasma previously obtained from these individuals will be analyzed for HDL cholesterol, using the heparin-manganese chloride method. Plasmas from suitable controls will be similarly analyzed.

2. Determination of lipoprotein composition in normal and diabetic Indians.

   Assays of lipoprotein cholesterol and triglyceride are being continued in order to extend the above findings to other sex and age groups in the normal and diabetic population. In addition, lipoprotein composition is being quantified in recent onset diabetics during diet-induced remission of glucose intolerance, in order to determine if alterations in lipoprotein composition accompany this therapy.

   Free fatty acid studies are being extended to assay the levels of free fatty acids during OGTT's or IVGTT's. These data will be correlated with insulin levels during these procedures to explore the relation between free fatty acids and insulin levels in this population.

Publications: None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)

U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE
PUBLIC HEALTH SERVICE
**NOTICE OF INTRAMURAL RESEARCH PROJECT**

PROJECT NUMBER
Z01 AM 54,017-01 DD

PERIOD COVERED
July 1, 1976 to September 30, 1977

TITLE OF PROJECT (80 characters or less)

Effects of weight-reduction on glucose tolerance and gluco-regulatory hormones

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT


PI: P.H. Bennett, Acting Chief PCRS, NIAMDD
Other: P.J. Savage, Staff Physician SFSS, NIAMDD
L.J. Bennion, Staff Physician PCRS, NIAMDD
D. Mott, Staff Fellow PCRS, NIAMDD


COOPERATING UNITS (if any)

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A.H. Rubenstein, Univ. of Chicago, School of Medicine, Chicago

LAB/BRANCH
Digestive Diseases Branch

SECTION
Phoenix Clinical Research Section

INSTITUTE AND LOCATION
NIAMDD, NIH, Phoenix, Arizona 85014

| TOTAL MANYEARS: | PROFESSIONAL: | OTHER: |
|---|---|---|
| 0.9 | 0.5 | 0.4 |

CHECK APPROPRIATE BOX(ES)

☒ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)


Adults with recent onset of symptomatic, but ketosis resistant diabetes mellitus were studied with oral and intravenous glucose tolerance tests, arginine infusions and circulating mononuclear cell insulin receptor assays. Subjects were then placed on a weight reduction diet. They were restudied every 5 weeks to determine the degree of reversibility of changes in glucose and gluco-regulatory hormones in diabetes.




FORM 2040
(Rev. 10-76)

Project Description:

Objectives: Many components of the natural history of diabetes remain to be described. A major difficulty is the absence of sequential data on individuals moving from the normal to the diabetic state. Even in populations with a high prevalence of diabetes our present inability to identify subjects who will rapidly become diabetic means that large numbers of subjects would have to be studied for many years.

The study of recent onset obese diabetics appears likely to reveal much about the interrelationship of glucose tolerance and gluco-regulatory hormone levels in a much shorter period of time. If normal glucose tolerance can be achieved by weight reduction many comparisons can be made between such subjects and subjects who have never had abnormal glucose tolerance.

The purposes of the study are:

1. To measure changes in insulin secretion which occur during the improvement of glucose tolerance which is known to occur with weight reduction in obese diabetics.

2. To measure changes in glucagon secretion which occur during improvement in glucose tolerance to determine whether the hyperglucagonemia seen in diabetes is reversible.

3. To determine both acute and chronic changes in insulin receptor concentrations and affinity and to evaluate whether they are solely secondary to dietary changes or affected by the presence or absence of the diabetic state.

4. To determine changes in lipoprotein concentrations and fractions which occur during improvement of glucose tolerance.

Methods Employed: Fifteen obese diabetics with onset of glucose intolerance within the past 4 years, who have never been treated with insulin and whose 2 hour plasma glucose levels are $\geq$250 mg/dl will be admitted to the clinical research center. Fifteen similarly obese non-diabetics will be admitted as controls for the effects of weight reduction itself.

Subjects will initially be placed on a weight maintaining 300 gm carbohydrate diet and have oral and intravenous glucose tolerance tests, arginine infusions with measurement of insulin and glucagon responses and measurement of insulin receptor binding to circulating mononuclear cells. Subjects will then be placed on a 500 calorie 50 gm carbohydrate diet to induce rapid weight loss. Every fifth week subjects will be refed with a 1500 calorie 300 gm carbohydrate diet and after 4 days on the diet the initial series of tests will be repeated. Studies will be terminated when glucose tolerance stabilizes at abnormal levels or is reduced to normal levels.

Major Findings: To date 6 diabetic and 6 non-diabetic subjects have been studied. Partial results on the oral glucose tolerance tests are shown in Table 1 for glucose and insulin levels:

Table 1

| | Plasma Glucose (mg/dl) | | | Serum Insulin (μU/ml) | | |
|---|---|---|---|---|---|---|
| Time | 0 | 1/2 hr | 2 hr | 0 | 1/2 hr | 2 hr |
| Diabetics | | | | | | |
| Initial | 281±26 | 341±30 | 444±39 | 23±3 | 26±1 | 26±5 |
| Final | 102±6<br>p<0.001 | 161±10<br>p<0.001 | 212±17<br>p<0.001 | 19±3<br>ns | 63±18<br>p<0.05 | 103±21<br>p<0.05 |
| Normal | | | | | | |
| Initial | 87±2 | 145±5 | 128±14 | 22±4 | 249±64 | 140±39 |
| Final | 86±2<br>NS | 135±9<br>NS | 110±6<br>NS | 10±3<br>p<0.05 | 96±22<br>p<0.01 | 94±17<br>NS |

It thus appears that the improvement in glucose tolerance is accompanied by a recovery of insulin secretory response. A similar pattern of increasing insulin levels in the diabetics and decreasing insulin levels in the non-diabetics was found in the intravenous glucose tolerance tests.

In contrast, insulin secretion of the diabetics during arginine infusions was relatively normal initially and improved only minimally after improvement of glucose tolerance. These findings indicate that a selective defect of insulin secretory response to a glucose stimulus occurs early in the course of diabetes and that at least part of this defect is reversible.

Results of other studies are not yet available.

Significance to Biomedical Research: These studies appear to have a good prospect of differentiating the reversible and irreversible abnormalities that characterize diabetes mellitus. It is possible that the findings can be utilized to improve our methods of treating maturity-onset diabetes.

Proposed Course: Additional subjects will be studied to further clarify the sequence of changes as recovery occurs. We also hope to study subjects with minimal degrees of glucose intolerance and some ketoses resistant subjects who have required insulin therapy to control symptoms.

Publications: None

| SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space) | U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT | PROJECT NUMBER Z01 AM 54,018-01 DD |
|---|---|---|

PERIOD COVERED

July 1, 1976 to September 30, 1977

TITLE OF PROJECT (80 characters or less)

Characterization of Metabolic and Morphologic Abnormalities in Diabetes

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

| | | |
|---|---|---|
| PI: | P.H. Bennett, Acting Chief | PCRS, NIAMDD |
| Other: | P.J. Savage, Staff Physician | SFSS, NIAMDD |
| | L.J. Bennion, Staff Physician | PCRS, NIAMDD |
| | P. Gorden, Senior Investigator | D, NIAMDD |
| | J. Roth, Chief | D, NIAMDD |
| | B. Howard, Staff Fellow | PCRS, NIAMDD |
| | D. Mott, Staff Fellow | PCRS, NIAMDD |

COOPERATING UNITS (if any)

R.H. Unger, Univ. of Texas. Southwestern Medical School, Dallas
M.D. Siperstein, Univ.of California Medical School, San Francisco
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S.L. Aronoff, Washington Univ., School of Medicine, St. Louis

LAB/BRANCH

Digestive Diseases Branch

SECTION

Phoenix Clinical Research Section

INSTITUTE AND LOCATION

NIAMDD, NIH, Phoenix, Arizona 85014

| TOTAL MANYEARS: | PROFESSIONAL: | OTHER: |
|---|---|---|
| 0.9 | 0.5 | 0.4 |

CHECK APPROPRIATE BOX(ES)

☒ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Age, sex and weight matched groups of genetically normal, prediabetic and diabetic Pima Indians and normal Caucasians were examined by means of oral and intravenous glucose tolerance tests, arginine infusions and circulatory mononuclear cell insulin receptor assays, measurement of thickness of muscle capillary basement membrane and urinary protein profiles to determine whether abnormalities of insulin, glucagon secretion or other measurements are characteristic of the prediabetic and diabetic state.



PHS-6040

Project Description:

This investigation is a continuation and expansion of the previous projects: 1) Evaluation of the Prediabetic and Diabetic State in the Pima Indians (Z01 AM 69,003-03 EFSB) 2) Evaluation of the Prediabetic State in the Pima Indians - Caucasian Controls (Z01 AM 69,004-03 EFSB) 3) Evaluation of Basement Membrane Thickness in Diabetic Pima Indians (Z01 AM 69,005-03 EFSB).

Objectives: Many components of the natural history of diabetes remain to be described. A prospective study of genetically suspect prediabetic subjects (defined as the non-diabetic offspring of two diabetic parents) appears to be the most direct and reliable method of describing the natural history of the disease.

Study of the prediabetic will enable us to better understand the interrelationship of glucose levels with insulin and glucagon levels, an area which has remained controversial. These studies will also clarify the relationship of hyperglycemia to microangiopathy. Detailed studies of urinary protein abnormalities and muscle capillary basement membrane thickness should clarify the history of some of the earliest physical abnormalities found in diabetes. In addition the study was designed to clarify whether technical variations are responsible for the conflicting findings of Siperstein and Williamson regarding the time capillary basement membrane thickening is first seen.

The purposes of the study are:

1. To determine whether there are abnormalities in carbohydrate metabolism gluco-regulatory hormone secretion, urinary protein excretion or capillary basement membrane thickening in non-diabetic Pima Indians compared with non-diabetic Caucasian controls.

2. To determine if there are differences in these variables between Pima Indians with 2 parents with diabetes and those with 2 non-diabetic parents.

3. To compare the two major techniques of basement membrane measurement.

4. To determine if capillary basement membrane thickening is related to the duration of carbohydrate intolerance and to the complications of diabetes.

5. To initiate a long term prospective study of prediabetes.

Methods Employed: Four groups were selected for study - Pima Indian non-diabetic offspring of two diabetic parents, non-diabetic offspring of two non-diabetic parents, subjects with long and short duration of documented diabetes and non-diabetic Caucasians with two non-diabetic parents. The non-diabetic groups were matched for age, sex and percent desirable weight.

Following dietary preparation all subjects were admitted to the Clinical Research Center for testing. Tests included an oral glucose tolerance test,

intravenous glucose tolerance test, cortisone glucose tolerance test, an arginine infusion, a quadriceps needle muscle biopsy, fundus photographs and 24-hour urine collection for determination of specific urinary proteins by immunodiffusion.

Two sub-groups of diabetics were examined a) long-term diabetics, defined as subjects with fasting plasma glucose $\geq$140mg/100ml or a two hour post-carbohydrate load glucose >300 mg/100ml and >10 years duration with and without retinopathy, and b) short-term diabetics, defined as subjects who have had a documented marked deterioration in their oral glucose tolerance over the last 5 years from a two hour post-carbohydrate load plasma glucose $\leq$140 mg/100ml to a two hour plasma glucose $\geq$300 mg/100ml.

The very high prevalence of diabetes in the Pima people, while providing a very large group of genetically prediabetic individuals, raised a problem of finding individuals who are truly normal. Genetically normal Pima offspring were selected from phenotipically normal parents 45 years of age or older. It is conceivably possible, however, that with the high prevalence of diabetes in the tribe, virtually all the Pimas could carry the diabetic genome.

Because of this possibility, a group of nondiabetic Caucasians with nondiabetic family histories were examined as an additional control group to compare with the "normal" Pimas.

Results: The pattern of insulin response to oral and intravenous glucose has been claimed to be characteristic of diabetes and prediabetes. While insulin responses to oral glucose were reduced in subjects with 2 hour glucose levels over 225mg/dl and the acute phase insulin release was absent in all diabetics there was evidence of hyperinsulinemia in the Pima nondiabetics compared to the Caucasians. The normal and pre-diabetic Indians had fasting and stimulated insulin levels during all the tests 2-3 times greater than those on the Caucasians. Differences in insulin levels between the two races could not be explained by differences in glucose level, age or obesity. These studies raised the possibilities of either 1) extra pancreatic insulin resistance or 2) an abnormality of biological effectiveness of Pima insulin.

The pattern of glucagon response in the non-diabetic Pimas was similar to that found in the Caucasians. The "prediabetic" and "normal" Pimas had essentially identical glucagon responses. The glucagon response of diabetic Pimas was increased during arginine infusions to levels previously described in Caucasian diabetes. The suppression of glucagon secretion by I V glucose was found to be abnormal in Pima diabetics.

Urinary macromolecular protein excretion was examined in each of the four groups as a sensitive index of the presence of renal disease. To determine if glomerular basement membrane involvement occurs prior to onset of glucose intolerance or early in the course of diabetes 24 hr. urines were collected from 33 prediabetic, 25 normal, 31 diabetic Pima

Indians and 25 normal Caucasians, and concentrated by ultrafiltration and collodion bag dialysis 1-600x. Daily excretion (Mean ± SEM) of six proteins 1) $A_1$ acid glycoprotein, 2) CG globulin, 3)$A_1$ antitrypsin, 4) Albumin, 5) Transferrin, 6) IgG, ranging in molecular weight from 44,000 ($A_1$ acid GP) to 150,000 (IgG) daltons, was assayed by radial immunodiffusion and expressed as mg/24 hrs. The significance of differences was determined by analysis of variance and Duncan's multiple range test.

| | | Indians | | |
|---|---|---|---|---|
| Protein | Normal Caucasian | Normal | Pre-Diabetic | Diabetic |
| 1) | 0.57±.13 | 1.64±.59 | 1.44±.32 | 7.95±2.44* |
| 2) | 0.03±.01 | 0.02±.01 | 0.02±.005 | 0.63±0.27* |
| 3) | 0.23±.06 | 0.23±.09 | 0.19±.04 | 2.67±1.27# |
| 4) | 11.50±2.0 | 10.43±1.9 | 10.18±1.4 | 102.85±39.6# |
| 5) | 0.38±.07 | 0.43±.22 | 0.34±.06 | 8.44±3.77* |
| 6) | 1.82±.25 | 1.88±.43 | 2.09±.33 | 21.54±7.33+ |

*p<.01, #p<.05, +p<.001

Excretion of each of these 6 proteins was similar in prediabetic and normal Indians and normal Caucasian, but was significantly higher in diabetics. Eight of 10 diabetics of less than 2 years duration excreted more of the proteins than any of the prediabetic, normal or normal Caucasian. No evidence of changes in the renal-vascular permeability of these proteins was found prior to the onset of glucose intolerance, but changes were frequently demonstrable in diabetics of short duration.

Results of muscle capillary basement membrane studies are presently being analysed. Additional prediabetic and normal Pimas above age 30 were biopsied this year to clarify effects of aging on capillary basement membrane thickness. Results are presently available from analyses of data on 17 of 35 prediabetic, 18 of 26 normal and 25 of 33 diabetic Pimas and 20 of 34 normal Caucasians. Measurement of capillary basement membrane thickening in normal Indians and Caucasians indicated no consistent racial variation in basement membrane thickness. Capillary basement membrane measurements by both methods were thickened in the diabetics ($p<0.05$) and were significantly correlated with the duration of diabetes ($p<0.05$). The Siperstein method appeared more sensitive in detecting diabetics.

Significance to Biomedical Research and the Program of the Institute. These studies are providing information on issues which have been controversial in diabetes research for over a decade. They are making it possible to systematically approach the cause of diabetes in the Pima, a group with the highest known prevalence of maturity onset diabetes.

Proposed Course: Subjects will be recalled every two years for an additional series of tests. This should enable careful documentation of the natural history of diabetes. Additional studies will be conducted to evaluate insulin resistance in hyperinsulinemic Pimas and to evaluate the

biological activity of Pima insulin.

Publications:

Aronoff, S.L., Bennett, P.H., Rushforth, N.B., Miller, M., and Unger, R.H.: Normal glucagon response to arginine infusion in prediabetic Pima Indians. J. Clin. Endocinol. Metab. 43:279-286, 1976.

Aronoff, S.L., Bennett, P.H., Rushforth, N.B., Miller, M., and Unger, R.H.: Arginine stimulated hyperglucagonemia in diabetic Pima Indians. Diabetes 25:404-407, 1976.

Bennett, P.H., Aronoff, S.L., and Unger, R.H.: Evidence for an insulin-independent alpha cell abnormality in human diabetes. Metabolism 25, Suppl 1, 1527-29, 1976.

Aronoff, S.L., Bennett, P.H., Gorden, P., Rushforth, N., Miller, M.: Unexplained hyperinsulinemia in normal and prediabetic Pima Indians compared to normal Caucasians. Diabetes (in press).

Aronoff, S.L., Bennett, P.H., and Unger, R.H.: Immunoreactive glucagon responses to intravenous glucose in prediabetes and diabetes among Pima Indians and Normal Caucasians. J. Clin. Endocrinol. Metab. 44:968-972, 1977.

NIH Contract No. NO1-AM-6-2219 Starting date: September 30, 1976
Expiration date: September 25, 1978

Contractor: University of Texas Health Science Center
Southwestern Medical School

Money allocated: $360,541.00 Man-years purchased: 13

This contract is for extension of the studies carried out under the NIH project Z01-AM-54018-01, Characterization of Metabolic and Morphologic Abnormalities in Diabetes.

Work to date has primarily been comprised of consultations on studies of diabetes and lipid metabolism and the measurement of glucagon, insulin, lipids and lipoproteins on samples obtained by NIH personnel at the Phoenix Clinical Research Center.

Proposed Course: Additional studies are planned utilizing contract personnel working both in Phoenix and in Dallas to elucidate further the importance of glucagon in human diabetes mellitus. These will include further investigations of the relation of glucagon and insulin levels, attempts to restore glucagon secretion to normal by aggressive therapy of diabetes and the use of somatostatin and its analogues to investigate effects of glucagon suppression. In addition further morphological studies of diabetic pathology utilizing electron microscopy are planned. Genetic studies on lipid metabolism in diabetes and studies of lipoprotein turnover and interrelationships are about to begin.

| SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space) | U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT** | PROJECT NUMBER ZO1 AM 54,019-01 DD |
|---|---|---|

PERIOD COVERED
July 1, 1976 to September 30, 1977

TITLE OF PROJECT (80 characters or less)

Cellular Metabolism in Diabetic and Nondiabetic Subjects

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

| | | |
|---|---|---|
| PI: | P.H. Bennett, Acting Chief | PCRS, NIAMDD |
| Other: | B.V. Howard, Staff Fellow | PCRS, NIAMDD |
| | D. Mott, Staff Fellow | PCRS, NIAMDD |
| | P.J. Savage, Staff Physician | SFSS, NIAMDD |
| | L.J. Bennion, Staff Physician | PCRS, NIAMDD |

COOPERATING UNITS (if any)

R.H. Williams, Professor of Medicine, University of Washington

LAB/BRANCH
Digestive Diseases Branch

SECTION
Phoenix Clinical Research Section

INSTITUTE AND LOCATION
NIAMDD, NIH, Phoenix, Arizona 85016

| TOTAL MANYEARS: | PROFESSIONAL: | OTHER: |
|---|---|---|
| 0.8 | 0.4 | 0.4 |

CHECK APPROPRIATE BOX(ES)

☒ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Elevated insulin levels in Pima Indian adults compared to weight matched Caucasian controls suggest that cellular changes external to the pancreas may be important in the pathogenesis of diabetes mellitus. The aim of this project is to study several aspects of insulin function in cells obtained from normal and diabetic Pima Indians, in order to determine if diabetes mellitus is expressed in genetic differences at the cellular level. Insulin binding and its relation to insulin levels is being assessed in circulating monocytes from normal and diabetic individuals. In addition, fibroblast cultures are being established from normal and diabetic Pima Indians. Parameters of cell growth are being compared in the normal and diabetic cells, and insulin binding is being studied. In addition, cellular metabolism and transport functions are being quantified in the presence and absence of insulin, in order to determine if cellular abnormalities in insulin response occur in individuals with diabetes.

Project Number: This project has been transferred to PCRS from SFSS and was formerly described as Z01 AM 69,012-01

Objectives:

1. Insulin receptors in circulating monocytes. Insulin levels in non-diabetic Pima subjects are elevated when compared to weight matched Caucasian controls. This could be because of a decreased number of cellular insulin receptors, abnormal affinity of Pima cellular receptors for insulin, an abnormality in the insulin secreted by Pima subjects or antibody to the insulin or receptor site. Studies are in progress to evaluate these possibilities by measuring insulin receptor concentrations and affinity in weight matched genetic normal and genetic prediabetic Indians, diabetic Indians and Caucasians with normal glucose tolerance and no family history of diabetes.

2. Fibroblast culture and study: Previous reports of decreased cell viability and impaired protein synthesis cultured fibroblasts from diabetics indicate the fibroblast is useful in the study of diabetes. Fibroblast cultures have been established from skin biopsies of genetic normal, genetic prediabetic and diabetic Pima Indians and normal Caucasians. Parameters of growth will be compared in cells from normal and diabetic Pimas in order to determine if adult-onset diabetes is reflected in cellular growth changes similar to those reported for cultures derived from juvenile-onset diabetics in the Caucasian population. Assays for cell responses to insulin will be established using normal cells, and the sensitivity to insulin and magnitude of response will be compared in the diabetic cells. In addition insulin binding will be assessed in these cultures, and the relation between insulin binding and its metabolic effects will be explored.

Major Findings:

1. Insulin receptors in monocytes. Insulin binding has been measured in circulating mononuclear cells from Caucasians and normal, prediabetic and diabetic Pima Indians. Although fasting insulin levels are significantly higher in the Indians, maximum specific binding was similar in all groups. Whereas receptor number correlated inversely with fasting insulin levels in the Caucasians, receptor number was not significantly related to insulin levels in any of the Pima groups.

TABLE I

INSULIN BINDING IN MONOCYTES - CORRELATION WITH FASTING INSULIN LEVELS

(mean ± SEM)

| | N | Max. Specific Insulin Binding (%/4x10$^6$ cells) | Fasting Insulin (μU/ml) | r | p |
|---|---|---|---|---|---|
| CAUCASIANS | 18 | 6.3 ± 0.3 | 16.9 ± 3.2 | -0.45 | <0.05 |
| INDIAN | | | | | |
| Non-diabetic | 24 | 6.3 ± 0.4 | 27.8 ± 2.7 | -0.29 | n.s. |
| Normal | 13 | 6.9 ± 0.5 | 26.9 ± 3.4 | -0.24 | n.s. |
| Prediabetic | 11 | 5.8 ± 0.5 | 32.2 ± 4.2 | -0.31 | n.s. |
| Diabetic | 6 | 5.1 ± 0.6 | 34.6 ± 7.4 | -0.12 | n.s. |

2. Fibroblast Studies. Fibroblast cultures from Caucasians and non-diabetic Pima Indians have been studied in order to define a consistently-measureable response to insulin. Radio-labelled glucose uptake has been measured in the presence and absence of insulin. Control experiments indicate that preincubation of cells in the absence of glucose for 12 to 18 hours results in a stimulation of the rate of glucose uptake, and an increase in the magnitude of response to insulin. When concentrations of glucose in the preincubation were decreased from 100 1.0 μg/ml, response to insulin increased 3 to 6 fold. Titration of the concentration of insulin required for stimulation of glucose uptake indicated half maximum stimulation at approximately 2 x $10^{-9}$M.

   Insulin binding has also been measured after pre-incubation for 17 hours in the absence of serum. Data indicate a $K_d$ of 1.6 x $10^{-9}$M, and an average $R_o$ of 6900 sites/cell in fibroblasts assayed in suspension at 15°C.

Significance to Biomedical Research and the Program of the Institute.

1. Insulin receptors in monocytes. The measurement of insulin receptors will increase understanding of the hyperinsulinemia described in Pima subjects. Identifying the cause of hyperinsulinemia may be important in understanding the excessive risk of diabetes in Pima subjects.

2. Fibroblast studies: Detailed studies of fibroblast growth should yield important information on whether possible genetic defects in adult-onset diabetes are reflected in altered parameters of cell growth. Assay of insulin binding and cell response to insulin should yield information on the mechanism of insulin action at the cellular level. Moreover, comparisons of normal and diabetic cells will indicate if defects are reflected at the cellular level in this disease.

Proposed Course:

1. Insulin receptors in monocytes. Insulin receptor assays in circulating monocytes are continuing in additional samples from Pima diabetics and non-diabetics in order to define more precisely the parameters of binding and receptor number in these groups. In addition, binding is being assessed in recent onset diabetics before and after diet-induced remission of carbohydrate intolerance in order to determine the effects of changing insulin levels on binding affinity and receptor number.

2. Fibroblast studies. Parameters of growth are being systematically quantitated in fibroblast cultures derived from carefully defined normal and diabetic Pima Indians. These include plating efficiency, population doubling time, confluent cell density, and total number of population doublings. These data will be compared to similar data obtained from fibroblasts derived from normal Caucasians and juvenile-onset diabetics.

   Glucose uptake and its stimulation by insulin is being compared in cultures from normal and diabetic Indians. Effects of growth rate and population doublings are being evaluated so that the normal and diabetic cells can be compared in terms of their sensitivity and magnitude of response to insulin.

   Kinetics of insulin binding and receptor number in relation to insulin levels are being evaluated in control cultures from normal Indians and Caucasians. Binding parameters are being compared in cells from normal and diabetic Indians in order to assess if diabetes is reflected in cellular differences in binding of insulin.

Publications:

Howard, B.V., delaLlera, M., Howard, W.J.: A new method for the establishment of diploid fibroblast cell cultures from human foreskins. Proc. Soc. Exp. Biol. Med. 153:280-283, 1976.

Howard, B.V.: Acetate as a carbon source for lipid synthesis in cultured cell. Biochim. Biophys. Acta, 1977 (in press).

Howard, B.V.: Uptake of very low density lipoprotein triglyceride by bovine aortic endothelial cells in culture. J. Lipid Res. 1977 (in press).

ANNUAL REPORT - 1976-1977

CLINICAL ENDOCRINOLOGY BRANCH

Dr. Harold Edelhoch received the Distinguished Service Award of the Public Health Service in 1977. He also served as Visiting Professor at the University of Stellenbosch in South Africa for a 2-month period in the Department of Pharmacology. Dr. Jamshed Tata of the National Institute for Medical Research, Mill Hill, was Distinguished Visiting Scientist in CEB for 4 months. The laboratory was also host to Visiting Associates and Visiting Fellows from Belgium, Italy and Japan.

## I. Thyroid Biochemistry

### A. Thyroid Cell Membrane and Mechanism of TSH Action

TSH action on the thyroid cell is exerted through an interaction on the basal plasma membrane with the hormone receptor and with an effector system involving adenylate cyclase. In order to characterize further the components of this system, the adenylate cyclase which had been activated with guanylyl-5'-imidodiphosphate was solubilized by the use of a non-ionic detergent. About 25% of the catalytic activity was obtained in soluble form and some of its properties were defined. The enzyme is heterogeneous, and has an approximate molecular weight of 180,000. Unlike the *in situ* enzyme, the solubilized enzyme is not stimulated by prostaglandin $E_1$ or inhibited by membrane-reactive substances such as filipin, valinomycin, trifluoperazine and polylysine except at very high concentration. The inhibitors, which earlier studies showed were active at a locus between the TSH receptor and the enzyme, appear to require that the adenylate cyclase reside within the membrane matrix for inhibitory activity. (Wolff, Asbury)

Studies on the interaction between various gangliosides, TSH and the solubilized TSH receptor have been started. A technical problem related to the slow dissociation rate of ganglioside micelles is under investigation by the use of solvents which speed up the dissociation rate. These methods will be used to characterize the mechanism and consequences of the interaction between TSH and the thyroid cell membrane. (Edelhoch, Colonna)

### B. Iodination Reactions

Lactoperoxidase was employed as a model for the thyroid peroxidase, since the former is a readily available, soluble enzyme with very similar properties. Spectroscopic studies showed that cysteine and glutathione shift the Soret band of lactoperoxidase, a reflection of the binding of the thiols to the enzyme. It was also shown that glutathione or other sulfhydryl compounds enhance the binding of diiodotyrosine to the enzyme. These interactions will help to clarify the mechanism of thyroid hormone synthesis in which thyroid peroxidase plays a central role. (Cahnmann, Pommier).

C. Iodoproteins

The biosynthesis of the major thyroid iodoprotein, thyroglobulin, has been studied further in the guinea pig in order to define the origin of the 3 thyroglobulin chains found in this animal species. The kinetics of $^{3}H$-leucine incorporation was analyzed, and required a 2-compartment model representing rapidly-turning over and slowly-turning over thyroglobulin. The kinetics could not distinguish between 2 possibilities: that the 3 thyroglobulin chains were independently synthesized or that the 2 smaller chains arose from the larger one (MW = 295,000). The turnover of the large chain was much more rapid than the others. These findings confirm other studies which indicate that the bulk of the daily requirement for thyroid hormone originates from newly synthesized thyroglobulin rather than from older, more highly iodinated molecules. (Rall, Burkhardt)

D. Thyroxine Synthesis

The mechanism by which preferential coupling of certain iodotyrosines in thyroglobulin leads to thyroid hormone formation has eluded prior analyses. Previous work on thyroxine synthesis in random copolymers of lysine and tyrosine has been extended to polymers of known sequence. When tyrosyl residues were separated by 3 lysyl residues, as compared to 5 lysyls, the yield of thyroxine increased from 4.7% to 14% of incorporated iodine. Atomic models showed that the increased yield correlated with the ability of the phenyl rings to arrange themselves in antiparallel fashion, and thus facilitate the formation of the diphenyl ether bridge. (Cahnmann, Pommier, Nunez)

E. Thyroid Hormone Secretion

Microtubules in the thyroid cell take part in the process of endocytosis and thyroid hormone secretion. The interaction of the subunit, tubulin, with ligands which affect microtubule function have been studied. Two binding sites for vinblastine were found: a high affinity site which accounts for the depolymerization behavior of tubulin, and a low affinity site which accounts for aggregation behavior. Maytansin interacts with both vinblastine sites but does not prevent the aggregating effect of vinblastine. The binding parameters of podophyllotoxin, a competitive inhibitor of colchicine binding, have been defined. Tropolone, another competitive inhibitor of colchicine binding, has been shown to have no effect on podophyllotoxin binding. The data indicate that podophyllotoxin and colchicine each have at least 2 points of attachment to tubulin, one of which is shared. Lithium ion, an agent known to interfere with thyroxine secretion, promotes tubulin polymerization and protects microtubules against the depolymerizing effects of colchicine or vinblastine. (Wolff, Bhattacharyya, Cortese)

F. Thyroxine-protein Interactions

The chemical properties of the major transport protein for thyroid hormone in human plasma, thyroxine-binding globulin (TBG), has been under study.

Although it has approximately the same molecular weight as thyroxine-binding prealbumin (54,000), TBG differs markedly in other properties. Thus, it is composed of a single polypeptide chain, contains 23% carbohydrate, and has a single hormone-binding site. There is a C-terminal leucine and 10 sialic acid residues. TBG has a compact structure, half of which is equally distributed between α-helical and β-structures. A minor structural change occurs below pH 5, during which the hormone binding site is irreversibly destroyed. (Robbins, Edelhoch, Cheng, Gershengorn, Lippoldt)

The binding of thyroid hormone analogs by prealbumin has been studied by a fluorescence method using ANS. Although thyroxine and some of its analogs quenched ANS fluorescence only through a displacement reaction, desamino analogs had an additional quenching effect on ANS still occupying the second binding site. Equations were developed to measure the binding affinity of each type of ligand by fluorescence measurement. (Robbins, Edelhoch, Cheng)

The effect of thyroid hormone on the synthesis and metabolism of TBG was studied in Rhesus monkeys, a species in which TBG closely resembles the human protein. Purified monkey TBG labeled with $^{125}I$ was employed. The hormone caused a decrease in the distribution volume and an increase in disappearance rate which was proportional to the amount of hormone administered. In contrast, there was a biphasic effect on TBG production - an increase at a physiological hormone level and a decrease at a hyperthyroid level. In addition a transient fall in the TBG level in plasma was caused during induction of $T_3$ toxicosis. The findings explain observed alterations of TBG levels in hyper- and hypothyroid patients. (Robbins, Glinoer, Goldman)

G. Metabolism of Thyroid Hormones

Extrathyroidal monodeiodination of thyroxine is an important mechanism for both hormone activation and inactivation. This process was studied in monolayer cultures of monkey hepatocarcinoma cells. An active nonphenolic ring deiodinating system was found, resulting in "reverse" triiodothyronine (3,3',5'-$T_3$) production from $T_4$. This metabolic pathway was enhanced in starved cells, providing a possible explanation for the increased "reverse" $T_3$ levels seen in man after starvation or malnutrition. The lack of effect of starvation on phenolic ring deiodination in the cell cultures indicated that separate enzymes take part in these two processes. The cells produce sulfoconjugated 3,3'-diiodothyronine as the major product of $T_3$ metabolism, and also produces a minor metabolite, 3',5'-diiodothyronine, which has not been demonstrated previously. This *in vitro* system should prove useful in the study of thyroid hormone metabolism. (Robbins, Cahnmann, Sorimachi)

H. Studies in Thyroid Disease

Improved methods for thyroid cancer diagnosis have been under study. In the preoperative diagnosis of thyroid nodules, fine needle aspiration cytology was employed. Objective evaluation of the cytological findings gave a correct

diagnosis in 78% of cases classified as malignant, and 94% of those classified as benign. This procedure, therefore, is useful in assessing the need for surgery in high surgical risk patients and in selecting patients for thyroid suppression therapy. Although used widely in other countries, needle biopsy of the thyroid has not heretofore been popular in the United States.

Preliminary results of a study to evaluate the optimum time for testing radioiodine uptake in thyroid cancer metastases have suggested that a shorter period of hypothyroidism may be adequate. If confirmed in further studies, this will significantly reduce patient discomfort.

Lithium was shown in one patient to decrease the rate of release of $^{131}I$ from thyroid cancer metastases. This should provide a means to increase the therapeutic/toxic ratio of $^{131}I$ therapy for this disease. The theoretical benefit, however, needs careful evaluation since the patient developed severe bone marrow toxicity after $^{131}I$ was given.

Radioimmunoassays for serum thyroglobulin and for serum thyroxine-binding globulin were developed. The former was applied to the study of a population in the Marshall Islands which had been exposed to fallout radiation. This procedure can detect elevated levels of thyroglobulin in patients with thyroid cancer or other thyroid nodules. In the population under study no new cases of thyroid cancer were detected by this technique. The TBG assay was applied to a study of hepatocellular carcinoma, and showed a slight increase in serum TBG level in this disease.

Additional cases of hyperthyroidism due to inappropriate TSH secretion were studied. Patients with pituitary tumors had elevated alpha-subunit levels and autonomous TSH secretion, whereas patients with thyroid hormone "resistance" syndromes had appropriate alpha secretion and responsiveness of TSH to TRH and thyroid hormones. The heterogeous nature of this syndrome is evident. (Robbins, Weintraub, Goldman, Gershengorn, McClung)

## II. Polypeptide Hormones

### A. Structure

The thermodynamics of the trimerization of glucagon has been evaluated by circular dichroism measurements. The predominantly hydrophobic nature of this reaction has been demonstrated by the temperature and concentration dependence. The association is enthalpically driven, and in the analysis, the coil to helix transitions during trimerization had to be taken into account. (Edelhoch, Formisano, Johnson)

### B. Secretion and Metabolism

Dispersed cells from normal, hyperplastic and neoplastic rodent pituitary glands have been used to study subunit and TSH synthesis. In all cases, alpha subunit synthesis exceeds that of beta subunits. The ratio $\alpha/\beta$ is highest in tumors. Beta subunit production is rate-limiting and is preferentially regulated by TRH and by thyroid hormones. Heterogeneity of TSH and the

subunits has been observed both in the cells and in the culture medium and will be examined for possible precursor-product relationships. The correlation between these components seen in rodent cell cultures to the large form of TSH-β subunit observed in normal and neoplastic human pituitary will also be studied. (Weintraub, Rosen, Blackman, Gershengorn)

Radioimmunoassays, receptor assays and bioassays of thyrotropin releasing hormone have been developed to study the control of TRH secretion. Preliminary evidence suggests that there may be a disparity in the measured TRH among the 3 assays. (Weintraub, Marshall)

C. Pituitary Pathophysiology

Basal encephalocoele, a congenital herniation of the brain into the sphenoid sinus, has been studied in 3 patients. They show variable patterns of anterior pituitary hypofunction, presumably related to variable interruption of hypothalamic pituitary pathways and possibly to congenital midline defect. All patients with this disorder require careful evaluation of pituitary function. (Rosen, Lieblich)

D. Ectopic Polypeptide Hormone Syndromes

A study of the incidence and specificity of placental polypeptide hormones and other proteins in cancer markers has been continued. These proteins appear to distinguish cancers from benign tumors, and their concentrations in plasma often correlate with the response to therapy. Various permanent cell culture lines have been established to study the prevalence of these proteins and their subunits. These cells also show discordant ectopic protein production and will be used to study their synthesis.

*In vitro* translation of normal human placenta mRNA by wheat germ has been used to study the synthesized forms of human placental lactogen (hPL). From 70-90% of the immunoreactive hPL has a larger apparent molecular weight than hPL dimer and may be similar to other high molecular weight lactogens previously described *in vivo*. (Rosen, Weintraub)

III. Adrenal and Testis Cell Secretion

Steroidogenesis by adrenal tumor cells in culture is stimulated by a large number of adenosine containing compounds. This phenomenon appears to be due to the conversion of these compounds on the cell surface to a common active product, adenosine. Similar effects occur with a testicular Leydig cell tumor line. Adenosine appears to act on the cell surface through the cyclic AMP system, and its effects is prevented by adenosine deaminase. (Wolff)

IV. Studies on Cell Surface Protein (CSP)

CSP is a protein isolated from the plasma membrane of fibroblasts which appears to have a role in cell transformation. Studies on this protein have

revealed a monomer-dimer reaction related to disulfide bond formation, and both forms are present in the isolated material. The structure and stability of these proteins are under investigation. (Edelhoch, Johnson, Colonna)

V. Adenylate Cyclase of B. pertussis

Bordetella pertussis organisms produce a number of substances, some of which are biologically active in the host animal and some of which appear to be important in the metabolism of the bacteria. One component of the organism causes an increase in circulating insulin levels in the host animal. Another bacterial product, which causes dermonecrosis and death in mice and rats, was shown to be located intracellularly. The bacterial adenylate cyclase, which has been shown previously to be predominantly extracytoplasmic in this organism, demonstrates changes in its activity which are dependent upon phase and conditions of growth and the culture media being used. The adenylate cyclase and the factor which increases insulin levels have been shown to be present in some standard Pertussis vaccines. (Wolff, Hewlett)

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PERIOD COVERED

July 1, 1976 through September 30, 1977

TITLE OF PROJECT (80 characters or less)

Thyroxine-Protein Interactions

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

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| | J. M. Goldman | Clinical Associate | CEB NIAMDD |
| | H. Edelhoch | Senior Scientist | CEB NIAMDD |
| | J. Robbins | Chief, Clin. Endo. Br. | CEB NIAMDD |
| OTHER: | R. Lippoldt | Sr. Health Services Offcr. | CEB NIAMDD |
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Clinical Endocrinology Branch

SECTION

Endocrine Biochemistry

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NIAMDD, NIH, Bethesda, Maryland 20014

| TOTAL MANYEARS: | PROFESSIONAL: | OTHER: |
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| 2.0 | 1.7 | .3 |

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☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Thyroxine-binding globulin (TBG) was purified from human plasma and shown to have a molecular weight of 54000 and a carbohydrate content of 23%. Quantitative C-terminal amino acid analysis, peptide mapping and amino acid composition, and denaturation of the reduced, alkylated protein showed that TBG is a single polypeptide chain. The molecule has a single binding site for the hormone, which is irreversibly lost when TBG is acidified below pH 5. TBG is a compact, symmetric molecule containing about 25% α-helical and 25% β-structures.

The binding of thyroid hormone analogs by serum prealbumin (PA) was studied by a fluorescence method employing ANS. Analysis of binding required two models, since some analogs, but not others, caused quenching of ANS fluroescence while the chromophore occupied one of the two binding sites on PA.

The thyroxine analog 3,5-dinitro-3',5'-diiodothyropropionic acid was synthesized. Its suitability as a photoaffinity label for the thyroxine-binding site on prealbumin was investigated.

Project Description:

Thyroxine-binding globulin (TBG) was purified from human plasma by affinity, ion-exchange and gel filtration chromatography. The molecular weight was 54000 daltons, and carbohydrate accounted for 23% of the molecular weight. There were 10 sialic acid residues per molecule, and a single C-terminal leucine residue. Four lines evidence failed to demonstrate subunits, in contrast to findings in another laboratory: quantitative carboxy terminal amino acid analysis, peptide mapping and amino acid composition, treatment with dodecyl sulfate, and denaturation of the reduced, alkylated protein with guanidine. The single polypeptide chain of TBG contains one binding site for thyroxine.

The secondary and tertiary structure of TBG was investigated by circular dichroism and fluorescence properties. The relaxation time of TBG indicated that it is a compact, symmetric molecule, and the CD spectrum indicated that about 1/2 of the peptide groups are equally distributed in α-helical and β-structures. Acidification below pH 5 produced a minor structural change which resulted in the irreversible loss of its affinity for thyroxine.

The binding of thyroid hormone analogs by serum preablumin (PA) was studied by a fluorescence method employing anilinonaphthalene sulfonic acid (ANS). This is a more complex system than previously studied models, since PA has 2 identical binding sites for thyroxine exhibiting marked negative cooperativity. The binding of thyroxine, triiodothyronine and several analogs could be analyzed by a model in which the hormone competed with ANS for each of the binding sites. Desamino analogs, however, did not fit this model, since occupancy of one site by the analog quenched the fluorescence of ANS occupying the second site. Calculations by the appropriate model gave association constants which agreed closely with values obtained by equilibrium dialysis.

A search for a suitable photo affinity label to be used as a probe for thyroxine-binding sites led to the choice of 3,5-dinitro-3',5'-diiodothyropropionic acid. Synthesis proved to be difficult since the desiodo compound could not be iodinated, and was achieved by condensing 3,5-dinitrophloretic acid with 2,6-diiodohydroquinone. Strong binding to PA was shown by fluorescence titration. $^{125}$I-labeled compound was obtained by exchange labeling. Preliminary irradiation experiments using visible light showed covalent binding of ligand to protein.

Publications:

1. Robbins, J.: Thyroxine-Binding Proteins. In Jamieson, B. A. and Greenwalt, T. J. (Eds.): Trace Components of Plasma: Isolation and Clinical Significance. New York, Alan R. Liss, Inc., 1976, pp. 331-350

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PERIOD COVERED
July 1, 1976 through September 30, 1977

TITLE OF PROJECT (80 characters or less)
Nonenzymic Model Reactions for the Conversion of 4-Hydroxyphenylpyruvic Acid to Homogentesic Acid

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Terminated



PHS-6040

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PERIOD COVERED
July 1, 1976 through September 30, 1977

TITLE OF PROJECT (80 characters or less)

Model Reactions for the Synthesis of Thyroxine Residues in Thyroglobulin

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

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| OTHER: | J. Nunez | Director of Research | C.N.R.S. |

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LAB/BRANCH
Clinical Endocrinology Branch

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Endocrine Biochemistry

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20014

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

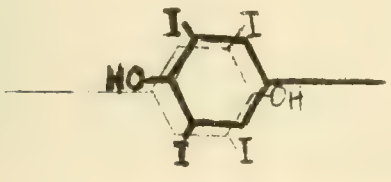
Peptides containing two Tyr residues were synthesized. They form thyroid hormone residues upon enzymatic iodination using thyroid peroxidase, and are, therefore, simple models for thyroglobulin.

Project Description:

Last year's report described a Lys-Tyr copolymer which, upon treatment with thyroid peroxidase in the presence of iodide, gave thyroxine ($T_4$) and triiodothyronine ($T_3$) residues in good yield. Attempts to use small Tyr-containing peptides as simpler models for thyroid hormone synthesis in thyroglobulin failed. Only small amounts of thyroid hormones were formed, if any.

However, it was found that $T_4$ and $T_3$ residues are formed in good yield when peptides containing more than one Tyr residue were used as substrates. Two peptides containing Lys residues and two differently spaced Tyr residues gave, upon enzymatic iodination, thyroid hormone residues in good yield. Yield and $T_4/T_3$ ratio depended on the distance between the two Tyr residues. With 3 Lys residues separating them, 2% of the incorporated iodine was in $T_3$ and 4.7% in $T_4$ ($T_4/T_3$ ratio, 2.35). With 5 Lys residues between the two Tyr residues, 1.6% was in $T_3$ and 14% in $T_4$ ($T_4/T_3$ ratio, 8.75).

Atom models show that both peptides can be arranged in such a configuration that the two aromatic rings of the Tyr residues are lying on top of each other. However, only in the peptide with 5 spacer Lys residues (I) are the two rings arranged in antiparallel fashion, which permits

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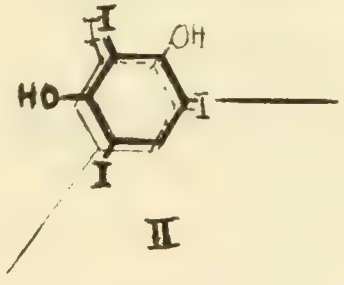


II

easy interaction with $T_4$ formation. In the peptide in which the Tyr residues are closer together (II) there is steric hindrance of the interaction caused by two iodine atoms in close proximity. This is in agreement with the lower $T_4/T_3$ ratio and with the lower total hormone yield obtained with the smaller peptide.

Publications:

None

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PERIOD COVERED

July 1, 1976 through September 30, 1977

TITLE OF PROJECT (80 characters or less)

Structure of Polypeptide and Protein Hormones and Lipoproteins

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

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| | M. Johnson | Staff Fellow | CEB NIAMDD |
| | G. Colonna | Guest Worker | CEB NIAMDD |
| OTHER: | R. E. Lippoldt | Sr. Health Services Officer | CEB NIAMDD |
| | L. Kohn | Section Chief | LBP NIAMDD |
| | I. Pastan | Laboratory Chief | LMBGY NCI |

COOPERATING UNITS (if any)

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LAB/BRANCH

Clinical Endocrinology Branch

SECTION

Endocrine Biochemistry

INSTITUTE AND LOCATION

NIAMDD, NIH, Bethesda, Maryland 20014

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SUMMARY OF WORK (200 words or less - underline keywords)

The predominately hydrophobic nature of the trimerization of glucagon has been demonstrated by measurements of the temperature and concentration dependence of its circular dichroism. Fluorescence and CD studies of the effects of Hofmeister salts and hydrophobic probes were used to further characterize the hydrophobic nature of the trimerization of glucagon.

Similar studies have been conducted on the cell surface protein isolated from fibroblasts by Yamada and Pastan. The protein appears to be very unstable and readily forms fibers, gels and films. The purified protein contains a mixture of monomer ($\sim 2 \times 10^5$ daltons) and dimer molecules.

We have begun a study on the effect of various gangliosides on the binding of TSH to its solubilized receptor. Measurements of the critical micelle concentrations of gangliosides are underway.

Project Description:

The thermodynamics of the trimerization of glucagon have been obtained from circular dichroism measurements as a function of concentration and temperature. According to a recent x-ray study of glucagon crystals, the nonpolar residues on the two ends of the glucagon chain are responsible for the association. The association is enthalpically driven between 5 and 45°C. In order to reconcile the interaction of nonpolar residues with a thermally controlled reaction it is necessary to take into account the effect of the nonpolar groups on the coil to helix transition in the trimerization. It can be shown that hydrophobic reactions can be exothermal if they include conformational changes. The description of hydrophobic reactions as entropically driven reactions can therefore be misleading. A better criterion is the negative change in heat capacity that accompanies all hydrophobic reactions.

The effect of several Hofmeister salts, phosphate, fluoride, etc., on the thermodynamics of glucagon association has been measured. It is known that Hofmeister anions affect nonpolar-water interactions. We have shown that the Hofmeister anion increases the enthalpy change and entropy change of the association. These trends are in accord with the usual interpretation of hydrophobic reactions.

We have begun to study the effect of various gangiosides on the binding of TSH to its solubilized receptor in collaboration with Dr. L. Kohn. We have made measurements by sucrose gradient and gel filtration in order to determine the critical micelle concentration (CMC) of different gangliosides. The measurements have been hampered by a very slow dissociation rate of the micelles to monomer. We are developing procedures of avoiding this problem by using solvents where the micelles are rapidly reduced to monomers.

We have made various physical (fluorescence, circular dichroism and sedimentation) measurements on the cell surface protein (CSP) isolated from fibroblasts by Yamada and Pastan. This protein disappears from the surface of these cells during transformation and its loss appears to be responsible for the altered shape and behavior of the transformed cells.

The purified protein contains a mixture of monomer and dimer molecules of molecular weight $\sim 2 \times 10^5$ and $4 \times 10^5$. The dimer is converted to the monomer by the reduction of some of its disulfide groups. The two molecules have the same or very similar properties by fluorescence and circular dichroism. It has been shown by Yamada and Pastan that their amino acid compositions are also similar. The protein appears to be very unstable and readily forms fibers, gels, and films on standing. We have attempted to assess the effects of pH, ionic strength and protein concentration on its structure and stability.

Publications:

1. Edelhoch, H.: Structure of Polypeptide Hormones. In Chen, R.F. and Edelhoch, H. (Eds.): Biochemical Fluorescence Concepts. New York, Marcel Dekker, Inc., 1976, pp. 545-571

2. Frenoy, J.-P., Bourrillon, R., Lippoldt, R. and Edelhoch, H.: Stability and subunit structure of human $\alpha_2$-marcoglobulin. J. Biol. Chem. 252: 1129-1133, 1977.

3. Ingham, K.C., Weintraub, B.D. and Edelhoch, H.: Kinetics of recombination of the subunits of human chorionic gonadotropin. Effect of subunit concentration. Biochemistry 15: 1720-1726, 1976.

4. Osborne, J.C., Jr., Palumbo, G., Brewer, H.B., Jr. and Edelhoch, H.: The thermodynamics of the self-association of the reduced and carboxymethylated form of ApoA-II from the human high density lipoprotein complex. Biochemistry 15: 317-320, 1976.

5. Ingham, K.C., Tylenda, C. and Edelhoch, H.: Structural studies of human chorionic gonadotropin and its subunits using tyrosine fluorescence. Arch. Biochem. Biophys. 173: 680-690, 1976.

6. Chen, R.F. and Edelhoch, H. (Eds.): Biochemical Fluorescence Concepts. New York, Marcel Dekker Inc., 1976, Vols. I and 2, 944 pp.

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PERIOD COVERED

July 1, 1976 through September 30, 1977

TITLE OF PROJECT (80 characters or less)

Thyroid Iodoproteins

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

| | | | | |
|---|---|---|---|---|
| PI: | A. E. Burknardt | Staff Fellow | CEB | NIAMDD |
| | J. E. Rall | Director, Intramural Research Programs | ODIRP | NIAMDD |
| | M. Berman | Senior Scientist | LTB | NCI |

COOPERATING UNITS (if any)

National Cancer Institute

LAB/BRANCH

Clinical Endocrinology Branch

SECTION

Endocrine Biochemistry

INSTITUTE AND LOCATION

NIAMDD, NIH, Bethesda, Maryland 20014

| TOTAL MANYEARS: | PROFESSIONAL: | OTHER: |
|---|---|---|
| 2.1 | 2 | .1 |

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☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The incorporation of $^{3}H$-leucine into guinea pig thyroglobulin was studied in vivo. The kinetic results thus obtained were tested against various models of synthesis by computer analysis. The only models yielding a satisfactory fit to the data contained two compartments for the synthesized thyroglobulin. The compartments differed in their turnover rate of thyroglobulin, the first compartment having a much faster turnover rate than the second. These results suggest that the bulk of the daily requirements of thyroid hormone in the peripheral tissue originates from newly synthesized thyroglobulin, not the older, more heavily iodinated thyroglobulin in the lumen.



FORM-6040

Project Description:

The goal of this project is to determine the nature and origins of the major thyroid iodoprotein, 19S thyroglobulin (MW 660,000).

Previous work had shown that guinea pig thyroglobulin, after reduction and alkylation, possessed three main protein chains (called A, B and C) when analyzed by SDS gel electrophoresis. The molecular weights of these chains are:

A - 295,000
B - 210,000
C - 110,000

While the relative amounts of the three chains present in thyroglobulin varies with the iodine content of the thyroglobulin, previous work in this laboratory demonstrated that iodination *per se* did not convert the largest species into the two smaller chains. This work also showed that the three chains were of *in vivo* origin and not degradative artifacts produced during the purification of thyroglobulin as was the case in other species.

The present work was designed to investigate the origins of the three polypeptide species of thyroglobulin by means of *in vivo* and *in vitro* kinetic studies. The kinetic data was then tested against various kinetic models using the SAAM program in an attempt to determine whether the smaller species are fragments of the largest or whether all three species are separately synthesized by the thyroid cell.

The procedure used was to inject guinea pigs i.p. with $^{3}H$-leucine and then to sacrifice the animals at intervals ranging from 1 hour to 14 days after the injection. The thyroglobulin from these animals was then purified and analyzed, after reduction, by SDS gel electrophoresis to determine the $^{3}H$-leucine content of the three species. The results, expressed as DPM of $^{3}H$/nmole of leucine in the protein, are:

| | Protein Species | | |
|---|---|---|---|
| Time | A | B | C |
| 1 hr | 20.6 | 2.9 | 2.0 |
| 3 | 68.6 | 6.1 | 2.0 |
| 5 | 65.7 | 9.0 | 3.2 |
| 10 | 172.6 | 18.3 | 5.4 |
| 22 | 249.8 | 40.3 | 14.9 |
| 60 | 225.8 | 56.3 | 28.6 |
| 96 | 146.5 | 63.5 | 29.8 |
| 120 | 131.1 | 74.2 | 31.9 |
| 192 | 128.6 | 91.4 | 54.7 |
| 336 | 85.8 | 78.4 | 53.7 |

The kinetic data fall into two patterns. The first is in the initial period after injection and lasts until about 96 hours. In this period there is a rapid increase in the $^{3}H$ content of the largest species, A, which reaches a maximum at about 22 hours. The $^{3}H$-leucine content of B and C also increase, though at rates slower than A. While the $^{3}H$-leucine content of A begins to decrease after 22 hours, that of B and C appear to reach a temporary plateau. The second pattern appears in the data from 96 hours to the end of the experiment at 336 hours. In this time interval, there is a decrease in the $^{3}H$-leucine content of A, though at a much slower rate than the decrease at the end of the first period. B and C begin to increase slowly at 120 hours, reach a maximum at about 200 hours, and then slowly decrease. In general, the turnover of $^{3}H$-leucine in all three species is much faster in the first period than in the second.

When this kinetic data was used to test the validity of kinetic models in the computer, simple models of leucine incorporation into A with subsequent degradation into B and C failed to fit the kinetic data. Likewise, simple models of separate synthesis of A, B and C also failed to fit the kinetic data. In order to obtain a satisfactory fit, it was necessary to postulate models in which two compartments for thyroglobulin are present. Leucine is initially incorporated into thyroglobulin in a compartment marked by a rapid turnover rate. In this compartment not only is there rapid incorporation of leucine into newly synthesized thyroglobulin, but also there is a rapid reabsorption of thyroglobulin back into the cell. Thyroglobulin reaches the second compartment only after passage through the first. Once in the second compartment, thyroglobulin is degraded slowly. If two compartments are used, both models of thyroglobulin synthesis will fit the kinetic data.

The two compartment models are compatible with the known physiology of the thyroid. Both the secretion of thyroglobulin into the lumen and its readsorption into the thyroid cell occur at the apical membrane. Thus newly secreted thyroglobulin would be the thyroglobulin most likely to be readsorbed and degraded. This model also suggests that the bulk of the daily requirement for thyroid hormone in the guinea pig originates from newly synthesized thyroglobulin, not the older, more heavily iodinated thyroglobulin that constitutes the central region of the follicle lumen.

Attempts to distinguish between the two potential models for thyroglobulin synthesis by means of *in vitro* synthetic studies using cycloheximide did not yield results that unequivocally favored one model or the other.

Publications:

None

| SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space) | U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT** | PROJECT NUMBER Z01 AM 45007-12 CEB |
|---|---|---|

PERIOD COVERED
July 1, 1976 through September 30, 1977

TITLE OF PROJECT (80 characters or less)
Protein Synthesis in the Thyroid Gland

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

| | | | |
|---|---|---|---|
| PI: | J. Robbins | Chief, Clin. Endo. Br. | CEB NIAMDD |
| | H. J. Cahnmann | Scientist Emeritus | CEB NIAMDD |

COOPERATING UNITS (if any)
None

LAB/BRANCH
Clinical Endocrinology Branch

SECTION
Endocrine Biochemistry

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20014

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| 0 | 0 | 0 |

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☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Inactive



PHS-6040

Project No. Z01 AM 45007-12 CEB

Project Description:

Inactive

Publication:

1. Izumi, M., Cahnmann, H. J. and Robbins, J.: Characterization of abnormal thyroglobulin in a transplantable rat thyroid tumor. Endocrinology 100: 1448-1460, 1977.

| SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space) | U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT** | PROJECT NUMBER Z01 AM 45008-04 CEB |
|---|---|---|

PERIOD COVERED
July 1, 1976 through September 30, 1977

TITLE OF PROJECT (80 characters or less)

Phosphoprotein Phosphatase of the Thyroid Gland

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

| | | | | |
|---|---|---|---|---|
| PI: | M. McClung | Clinical Associate | CEB | NIAMDD |
| | J. E. Rall | Director, Intramural Research Programs | ODIRP | NIAMDD |

COOPERATING UNITS (if any)

None

LAB/BRANCH
Clinical Endocrinology Branch

SECTION
Endocrine Biochemistry

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20014

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| 0 | 0 | 0 |

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Terminated

| SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space) | U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT | PROJECT NUMBER Z01 AM 45009-10 CEB |
|---|---|---|

PERIOD COVERED

July 1, 1976 through September 30, 1977

TITLE OF PROJECT (80 characters or less)

Studies in Thyroid Disease

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

| | | | |
|---|---|---|---|
| PI: | J. Robbins | Chief, Clin. Endocr. Br. | CEB NIAMDD |
| | B. D. Weintraub | Senior Surgeon | CEB NIAMDD |
| | J. Goldman | Clinical Associate | CEB NIAMDD |
| | J. Lieblich | Clinical Associate | CEB NIAMDD |
| OTHER: | E. W. Chu | Head, Cytopathology Sec. | LP NCI |

COOPERATING UNITS (if any)

National Cancer Institute

LAB/BRANCH

Clinical Endocrinology Branch

SECTION

Endocrine Biochemistry

INSTITUTE AND LOCATION

NIAMDD, NIH, Bethesda, Maryland 20014

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

To improve the methods for radioiodine testing in thyroid cancer, the effect of time after stopping thyroid hormone replacement was evaluated. An interval shorter than heretofore employed may be adequate.

In 50 consecutive thyroid nodules, fine-needle aspiration cytology was used to diagnose malignant lesions. A diagnosis of malignant by cytology was correct in 78% and a negative diagnosis was correct in 94%. In patients with suspicious cytology, 40% had cancer.

Lithium was shown in one patient to decrease the rate of release of $^{131}I$ from thyroid cancer metastases, and thus may provide a means to increase the therapeutic/toxic ratio in $^{131}I$ therapy for this disease.

Additional cases of hyperthyroidism due to inappropriate TSH secretion were studied. Patients with pituitary tumors had elevated alpha-subunit levels and autonomous TSH secretion, whereas patients with "resistance" syndromes had appropriate alpha secretion and responsiveness to TRH and thyroid hormones.

Project Description:

Thyroid Cancer Diagnosis: Improved methods for radioiodine testing of thyroid cancer are under study. In a continuation of previous work employing TRH stimulation of TSH release, data are being collected to compare $^{131}I$ uptake at short (2 wk) and long (4 wk) intervals after stopping triiodothyronine medication. Preliminary results indicate that the whole body scans at 2 wks are adequate for the detection of even minor foci of iodine uptake, but scans at 4 wks show a greater accumulation of iodine in some cases. If confirmed in a larger number of patients, the use of short term $T_3$ abstention will simplify testing for thyroid cancer metastases by avoiding symptomatic hypothyroidism.

Evaluation of fine-needle aspiration cytology in a consecutive series of 50 patients with hypofunctioning thyroid nodules was completed using an objective study design culminating in thyroidectomy and standard histological examination. The diagnosis by aspiration was correct in 78% of patients with a cytological diagnosis of carcinoma, and in 94% of patients in whom the cytology was negative. In cases with suspicious cytology, 40% were malignant. The procedure is therefore useful in assessing the need for surgery in high-risk patients and in selecting patients for thyroid suppression therapy.

Thyroid Cancer Treatment: The effect of lithium on iodine kinetics was studied in a patient with follicular thyroid carcinoma. Lithium decreased the release of $^{131}I$ from the tumor while having only a minimal effect on the rate of $^{131}I$ disappearance from blood. The increase tumor/blood ratio should enhance the therapeutic/toxic ratio of $^{131}I$ therapy. Since the patient experienced radiation toxicity following therapeutic radioiodine in the presence of lithium, the drug, although promising, must be used cautiously in future studies.

Measurement of Serum Thyroxine-Binding Globulin (TBG): A radioimmunoassay for serum TBG was developed, giving a normal value of $1.48 \pm 0.46$ mg/dl and a mean value in women (1.66) slightly higher than in men (1.37). In patients with hepatocellular carcinoma, the mean was slightly elevated ($2.10 \pm 1.29$) and was outside the normal limits in 2 patients.

Measurement of Serum Thyroglobulin: A radioimmunoassay for serum thyroglobulin (TG) was developed and was used in a population in the Marshall Islands previously exposed to fallout radiation and known to have a high incidence of thyroid disease. In most patients previously treated with thyroidectomy for benign or malignant thyroid nodules, the serum TG levels were undetectable. In exposed persons with no palpable thyroid nodules on Rongelap Island (high radiation exposure) and Uterik Island (low radiation exposure), the levels were similar to those observed in non-irradiated Americans. Since serum TG is known to be increased in thyroid cancer, the findings indicated that no further thyroid cancer was present in the population studied.

Inappropriate Secretion of TSH: We have studied serum from several additional cases of inappropriately high TSH secretion in the presence of elevated concentrations of total and free thyroid hormones. The new cases have confirmed our previous recognition of the heterogeneous origin of the syndrome: 1) pituitary tumors secreting TSH autonomously, 2) selective pituitary or generalized target organ resistance to the action of thyroid hormone. All of the new pituitary tumor cases have had greatly increased alpha subunit secretion, and autonomy of TSH and alpha response to TRH stimulation and thyroid hormone suppression. The new "resistance" syndromes have demonstrated appropriate alpha secretion and some responsiveness to TRH and thyroid hormones. In the tumor cases alpha subunit concentrations appear to correlate better with clinical response than do TSH levels. Currently studies are in progress to measure endogenous concentrations of TRH in these patients.

Publications:

1. Gershengorn, M. C., Larsen, P. R. and Robbins, J.: Radioimmunoassay for serum thyroxine-binding globulin: Results in normal subjects and in patients with hepatocellular carcinoma. J. Clin. Endocrinol. Metab. 42: 907-911, 1976.

2. Gershengorn, M. C., Wolff, J. and Larsen, P. R.: Thyroid-pituitary feedback during iodine repletion. J. Clin. Endocrinol. Metab. 43: 601-605, 1976.

3. Gershengorn, M. C., Izumi, M. and Robbins, J.: Use of lithium as an adjunct to radioiodine therapy of thyroid carcinoma. J. Clin. Endocrinol. Metab. 42: 105-111, 1976.

| SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space) | U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT** | PROJECT NUMBER Zo1 AM 45010-07 CEB |
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PERIOD COVERED
July 1, 1976 through September 30, 1977

TITLE OF PROJECT (80 characters or less)

Ectopic Placental Protein and Subunit Production by Tumors

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

| | | | |
|---|---|---|---|
| PI: | S. W. Rosen | Medical Director | CEB NIAMDD |
| | B. D. Weintraub | Sr. Surgeon | CEB NIAMDD |
| OTHER: | H. Sussman | Assistant Prof. Med | Stanford Univ. Med. Ctr. |
| | R. Kahn | Sr. Surgeon | DB NIAMDD |
| | P. Gorden | Medical Director | DB NIAMDD |
| | H. Brereton | Head, Rad. Med. Sec. | RB NCI |
| | J. Lieblich | Clinical Associate | CEB NIAMDD |
| | A. Rabson | Director, DCBD | OD NCI |
| | E. Heyderman | Staff Scientist | Ludwig Inst., London |
| | S. Aaronson | Head, Mol. Biol. Sec. | LRNATV NCI |
| | L. Broder | Assistant Professor | Univ. of Miami |
| | I. Kourides | Assistant Professor | Mass. Gen. Hosp. |

COOPERATING UNITS (if any)
Stanford University Medical Center, Palo Alto, CA; National Cancer Institute; Ludwig Institute, London, England; University of Miami; Massachusetts General Hospital, Boston, MA

LAB/BRANCH
Clinical Endocrinology Branch

SECTION
Endocrine Biochemistry

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20014

| TOTAL MANYEARS: | PROFESSIONAL: | OTHER: |
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| 2.1 | .8 | 1.3 |

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☐ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

We continue to study the incidence and specificity of placental proteins (chorionic gonadotropin and its free alpha and beta subunit, placental lactogen, and placental alkaline phosphatase) as cancer markers in vivo and in vitro. These proteins appear to distinguish cancers from benign tumors and their concentrations in plasma often correlate with the response to therapy. Various permanent cell lines have been established to study the prevalence of ectopic placental protein and subunit production, the regulation of placental protein synthesis in vitro and to characterize the properties of these materials as well as their in vitro response to chemotherapeutic agents. Immunocytochemical techniques have been developed to localize the cells responsible, in heterogeneous tumors, for ectopic placental protein and subunit production.



PHS-6040

Project Description:

A. In Vivo Studies

We continue to study the prevalence and specificity of neoplastic placental protein production viz. chorionic gonadotropin (CG), CG-β subunit, CG-α subunit, placental alkaline phosphatase (PAP), and placental lactogen (PL). The prevalence of these markers in a large series of patients with inoperable bronchial carcinoma is about 6%. There is discordance of marker production and no association of any marker with a particular cell type.

A similar discordance of marker production has been found in women with carcinoma of the breast. In these cases the prevalence appears, in preliminary analyses, to be higher, notably ectopic CG-α and PAP. In prostatic carcinoma, on the other hand, the prevalence of ectopic placental protein or subunit production was only about 2%.

We are analyzing sera from patients with a wide variety of cancers to see whether there is a significant difference in the ectopic production of pituitary glycoproteins and subunits relative to placental polypeptides.

B. In Vitro Production of Ectopic Placental Proteins

We are analyzing a variety of normal and neoplastic cell lines for ectopic placental protein production. Several hitherto unrecognized instances have been detected. These will be useful in assessment of the true prevalence of ectopic protein production (unbiased by complications inherent in vivo), in providing ready sources of ectopic peptides for characterization and in their potential for in vitro screening of chemotherapeutic agents.

Just as we found in vivo, ectopic production of placental proteins appears to be discordant in vitro. The corollary of this observation is that recognition of new instances of ectopic protein production should vary directly with the number of "markers" examined.

C. Cell Free Translation of Placental Protein Messenger RNA

We have previously shown that translation in wheat germ extracts of poly (A)-containing RNA isolated from human term placental results in the synthesis of two immunoreactive forms of hPL. The minor component co-electrophoreses on sodium dodecyl sulfate polyacrylamide gels with authentic hPL while the major component migrates with an apparent molecular weight about 3000 larger. In more recent investigations the cell-free products were purified by two methods: 1) precipitation with anti-hPL serum followed by dissociation and chromatography on Sephadex G-150 in 3M Gu·HCl, and 2) direct chromatography on Sephadex G-150 followed by location of hPL by precipitation with anti-hPL serum. In both cases 70-90% of immunoreactive hPL eluted with material of molecular weight greater than hPL dimer. The high molecular weight translated product bound to rat liver membranes, as did certain aggregates of iodinated lactogens, but not other

iodinated polypeptide hormones. Some bound translated product was displaced from lactogenic receptors by unlabelled monomeric hPL. This aggregated re-receptor-bindable translation product may be similar to high molecular weight lactogens previously described *in vivo*.

D. Immunocytochemical Studies

Using immunoperoxidase techniques we are attempting to localize sites of ectopic placental protein and subunit production. Preliminary data in the case of ectopic CG production by a gastric carcinoma show that only a small fraction of tumor cells stain for CG. This heterogeneity is being further studied.

Publications:

1. Lieblich, J.M., Weintraub, B.D., Krauth, G.H., Kohler, P.O., Rabson, A.S. and Rosen, S.W.: Ectopic and eutopic secretion of chorionic gonadotropin and its subunits *in vitro*: Comparison of clonal strains from carcinomas of lung and placenta. *J. Natl. Cancer Inst.* 56: 911-917, 1976.

2. Kourides, I.A., Weintraub, B.D., Rosen, S.W., Ridgway, E.C. and Maloof, F.: Secretion of alpha subunit of glycoprotein hormones by pituitary adenomas. *J. Clin. Endocrinol. Metab.* 43: 97-106, 1976.

3. Chou, J.-Y., Weintraub, B.D., Rosen, S.W., Whangpeng, J., Sussman, H.D., Haughom, J.R. and Robinson, J.C.: Synthesis of alpha subunit of human chorionic gonadotropin by presumptive HeLa cells. *In Vitro* 12: 589-594, 1976.

4. Lieblich, J.M., Weintraub, B.D., Rosen, S.W., Ghosh, N.K. and Cox, R.P.: Secretion of HCG-alpha subunit and HCG by HeLa strains. *Nature* 265: 746, 1977.

| SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space) | U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT** | PROJECT NUMBER Z01 AM 45013-05 CEB |
|---|---|---|

PERIOD COVERED
July 1, 1976 through September 30, 1977

TITLE OF PROJECT (80 characters or less)

TSH and Prolactin Studies; Subunits of Glycoprotein Hormones

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

| | | | |
|---|---|---|---|
| PI: | B. D. Weintraub | Sr. Surgeon | CEB NIAMDD |
| | S. W. Rosen | Medical Director | CEB NIAMDD |
| OTHER: | M. Blackman | Postdoctoral Fellow | CEB NIAMDD |
| | M. Gershengorn | Assistant Professor | N.Y.U. Med. Ctr. |
| | I. Kourides | Assistant Professor | Mass. General Hospital |
| | F. Maloof | Chief, Thyroid Unit | Mass. General Hospital |

COOPERATING UNITS (if any)

Harvard Medical School, Boston, MA; N.Y.U. School of Medicine, NY.

LAB/BRANCH
Clinical Endocrinology Branch

SECTION
Endocrine Biochemistry

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20014

| TOTAL MANYEARS: | PROFESSIONAL: | OTHER: |
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| 2.3 | 1.5 | .8 |

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Glycoprotein hormones and free subunits are secreted by animal and human pituitaries in vivo and in vitro. Normal thyrotropic cells synthesize α subunit in excess of β subunit, but the production of the latter is preferentially regulated by thyroid hormones. Pituitary tumors may secrete alpha subunits and TSH in an abnormal fashion and may be autonomous to normal regulatory factors. Heterogeneous forms of immunoreactive TSH and subunits have been identified, and certain forms of subunits have abnormal combining properties. Studies are in progress to further elucidate the mechanism of biosynthesis of glycoprotein hormones.



PHS-6040

Project Description:

A. Regulation of Biosynthesis and Secretion of TSH and Subunits by Dispersed Pituitary Cells.

We have continued studies concerning TSH and subunit biosynthesis using dispersed cells from normal, hyperplastic, and neoplastic (thyrotropic tumor) rodent pituitary glands. In all pituitary cells alpha subunit is synthesized in excess of beta subunit; the unbalanced alpha production is particularly marked in pituitary tumors where the alpha to beta production ratio exceeds 10. Beta subunit production appears to be the rate-limiting factor in TSH biosynthesis, and beta synthesis is preferentially regulated by thyroid hormones and thyrotropin releasing hormone. Gel chromatographic analysis of unlabeled hormones and subunits reveals heterogeneous immunoreactive forms in the cell and culture media. Such heterogeneity has been confirmed in the immunoreactive biosynthetic products of experiments employing labeled animo acid and carbohydrate precursors. Recently we have developed methods to augment the early cellular uptake of labeled precursors such that 5 to 10 minute "pulse" periods can be studied in relationship to later "chase" periods. Using these methods, studies are in progress to define the precursor-product relationships of the heterogeneous TSH and subunit species.

B. Combination of Subunits of Glycoprotein Hormones.

We have previously studied the detailed kinetics of combination for $\alpha$ and $\beta$ subunits derived from normal or neoplastic tissues. Various preparations of subunits differ greatly in the amount of physically combined (as defined by immunoassay and gel chromatography) as well as in the proportion of these combinations which have receptor-binding activity. Currently experiments are in progress to study the combining properties of the heterogeneous forms of labeled subunits biosynthesized by dispersed pituitary and ectopic cell lines.

C. Large Forms of Human Subunits.

We have continued to characterize the heterogeneous forms of subunits secreted by the normal human pituitary as well as pituitary and other tumors *in vivo* and *in vitro*. Recently we have shown that certain patients may secrete only a previously unrecognized large form (approximate MW 200,000) of TSH-$\beta$ immunoactivity. This material cannot be disaggregated by 6M guanidine, dithioerythritol, or ribonuclease and cannot be converted into a smaller form by exposure to low concentrations of trypsin. Other studies have revealed that the material is not related to an autoantibody or other TSH-binding protein. The significance of this large subunit form is not known, but may represent a normal precursor form that can be identified in the biosynthetic studies described above.

Publications:

1. Re, R.N., Kourides, I.A., Ridgway, E.C., Weintraub, B.D. and Maloof, F.: The effect of glucocorticoid administration on human pituitary secretion of thyrotropin and prolactin. J. Clin. Endocrinol. Metab. 43: 338-346, 1976.

2. Kourides, I.A., Weintraub, B.D., Martorana, M.A.L. and Maloof, F.: Alpha subunit contamination of human albumin preparations: interference in radioimmunoassay. J. Clin. Endocrinol. Metab. 43: 919-923, 1976.

3. Weintraub, B.D., Blackman, M. and Gershengorn, M.: Cellular Actions of Hypothalamic Hormones. In Bizollon, J. (ed.): Recent Data in Radioimmunoassay. Lyon, 1976, 191 pp.

4. Kourides, I.A., Re, R.N., Weintraub, B.D., Ridgway, E.C. and Maloof, F.: Metabolic clearance and secretion rates of subunits of human thyrotropin. J. Clin. Invest. 59: 508-516, 1977.

| SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space) | U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT** | PROJECT NUMBER Z01 AM 45014-06 CEB |
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PERIOD COVERED
July 1, 1976 through September 30, 1977

TITLE OF PROJECT (80 characters or less)

Adrenal Secretion

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: J. Wolff Medical Director CEB NIAMDD

COOPERATING UNITS (if any)

None

LAB/BRANCH
Clinical Endocrinology Branch

SECTION
Endocrine Biochemistry

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20014

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The role of adenosine in adrenal secretion has been investigated. Adenosine containing compounds that can be converted by a number of enzymes on the external surface of the Y-1 adrenal tumor cell membrane all stimulate steroidogenesis maximally probably through a receptor on the cell surface. The effect is inhibited by adenosine deaminase and theophylline. Adenosine also stimulates adenylate cyclase in membranes prepared from Y-1 cells but in this system it acts as only a partial agonist.



PHS-6040

Project No. Z01 AM 45014-06 CEB

Project Description:

Steroidogenesis by Y-1 adrenal tumor cells in culture is stimulated by ATP, adenyl-5'-yl imidodiphosphate (App(NH)p), adenosine 5'(β,γ-methylene)-triphosphate (App($CH_2$)p), ADP, AMP, NAD, FAD, and adenosine but not by adenine or other nucleoside triphosphates. ATP, App(NH)p, App($CH_2$)p, and adenosine are active in the micromolar range. Like adrenocorticotropic hormone (ACTH), the onset of stimulation is immediate and occurs to the same extent. Also active are 2'- and 5'-deoxyadenosine and 2'-chloro-adenosine whereas adenine xyloside, L-riboside, or arabinoside have very low activity. Stimulation is accompanied by rounding of the cells. Di-pyridamole, an inhibitor of adenosine transport, increased the response to low concentrations of adenosine, suggesting that adenosine acts externally.

Stimulation of steroidogenesis by adenosine or phosphorylated adenosine compounds fails to occur in the presence of crystalline adenosine deaminase, and the effect of the enzyme on adenosine, ATP, or NAD stimulation is reversed by the competitive inhibitor erythro-9[3-(nonane-2-ol)adenine. This suggests that the enzyme acts specifically on adenosine and a requirement for the conversion of the above compounds to adenosine seems probable. The inhibition of cAMP effects by adenosine deaminase suggests that some of its effects are also mediated by conversion to adenosine.

Similar stimulation is seen in I-10 Leydig tumor cells, but an ACTH-resistant mutant of Y-1 cells, called OS-3, is relatively resistant to adenosine.

Adenosine and 2-chloroadenosine stimulate adenylate cyclase in membranes from Y-1 and I-10 cells at concentrations slightly greater than are effective for steroidogenesis. Other nucleosides are ineffective. Like the $NH_2$-terminal 24 residues of adrenocorticotropic hormone ($^{1-24}$ACTH), the adenosine effect in Y-1 membranes is rapid and is on the $V_{max}$ intercept (versus ATP) and not on the $K_m$. In contrast to steroidogenesis, adenosine is only a partial agonist for adenylate cyclase. Its effect occurs in the presence of ITP, GTP, or guanyl-5'-yl imidodiphosphate (Gpp(NH)p).

Theophylline inhibits adenosine-stimulated steroidogenesis. Inhibition of adenylate cyclase occurs in the same concentration range but is of the mixed type.

Publication:

1. Wolff, J. and Cook, G.H.: Activation of steroidogenesis and adenylate cyclase by adenosine in adrenal and leydig tumor cells. J. Biol. Chem. 252: 687-693, 1977.

| SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space) | U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT** | PROJECT NUMBER Z01 AM 45015-07 CEB |
|---|---|---|

PERIOD COVERED

July 1, 1976 through September 30, 1977

TITLE OF PROJECT (80 characters or less)

Thyroid Plasma Membranes

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

| | | | |
|---|---|---|---|
| PI: | J. Wolff | Medical Director | CEB NIAMDD |
| | R. F. Asbury | Clinical Associate | CEB NIAMDD |

COOPERATING UNITS (if any)

None

LAB/BRANCH

Clinical Endocrinology Branch

SECTION

Endocrine Biochemistry

INSTITUTE AND LOCATION

NIAMDD, NIH, Bethesda, Maryland 20014

| TOTAL MANYEARS: | PROFESSIONAL: | OTHER: |
|---|---|---|
| 1.6 | 1.2 | .4 |

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Beef thyroid membrand adenylate cyclase has been solubilized by nonionic detergents and characterized. Differences from the membrane-bound enzyme are in pH optimum, response to stimulators and inhibition by a variety of compounds effective on membranes.

In addition, the relation between cationic and detergent properties of the basic protein melittin has been investigated. The detergent-like properties are the main determinants in the inhibitory effect on adenylate cyclase.

Project Description:

Bovine thyroid membrane adenylate cyclase activity has been solubilized using non-ionic detergent following activation with guanylyl 5'-imidodiphosphate (Gpp(NH)p). Optimum conditions for solubilization consistent with maintenance of catalytic activity have been defined. The yield of catalytic activity is approximately 25%. 80% of the membrane protein is solubilized. The specific activity of the solubilized preparation is 1300 pmol/10 min/mg protein compared to 2350 pmol/10 min/mg of membrane protein. The solubilized adenylate cyclase is heterogeneous, and studies are in progress to purify and characterize the individual components. The $K_m$ for adenosine triphosphate, and magnesium ion requirements of the solubilized preparation are similar to those of the membrane-bound enzyme. The solubilized activity is included in a Sepharose-6B gel, passes through a 0.22 micron Millipore filter, and is not sedimented by 300,000 xG for one hours. The approximate molecular weight is ∿180,000. Stimulation of the crude solubilized cyclase by additional Gpp(NH)p, or $F^-$ varies from preparation to preparation. Prostaglandin $E_1$ stimulates membrane-bound cyclase but has no effect on the solubilized enzyme. Many compounds previously shown to inhibit membrane adenylate cyclase including filipin, valinomycin, trifluoperazine, and polylysine, have no effect on the solubilized activity until much larger concentrations are added than are required for the membrane cyclase. Gramicidin-S and dextran sulfate are inhibitors of both membrane-bound and soluble cyclase. These studies suggest that many inhibitors interact with the membrane at a locus between the hormone receptor (shown previously) and the catalytic activity but require residence of the adenylate cyclase within the membrane matrix to be effective.

Melittin, a basic polypeptide from bee venom, inhibits basal and TSH-stimulated adenylate cyclase of beef thyroid membranes with a $K_i$ ∿10 μM. Although this property resides in the basic C-terminal and not the N-terminal portion of the molecule, inhibition is due primarily to its detergent-like nature rather than charge effects. There is also a small enhancing effect of both basal and TSH-stimulated adenylate cyclase of 0.3-3 μM melittin.

Publication:

1. Wolff, J.: Hormone and Antihormone Action at the Target cell. In Clark, J.H., Klee, W., Levitzki, A. and Wolff, J. (Eds.): Life Sciences Research Report 1. Berlin, Dahlem Konferenzen, 1976, vol. 3, 226 pp.

| SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space) | U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT** | PROJECT NUMBER Z01 AM 45016-07 |
|---|---|---|

PERIOD COVERED

July 1, 1976 through September 30, 1977

TITLE OF PROJECT (80 characters or less)

Thyroid Hormone Secretion

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

| | | | |
|---|---|---|---|
| PI: | J. Wolff | Medical Director | CEB NIAMDD |
| | B. Bhattacharyya | Visiting Associate | CEB NIAMDD |
| | F. Cortese | Postdoctoral Fellow | CEB NIAMDD |

COOPERATING UNITS (if any)

None

LAB/BRANCH

Clinical Endocrinology Branch

SECTION

Endocrine Biochemistry

INSTITUTE AND LOCATION

NIAMDD, NIH, Bethesda, Maryland 20014

| TOTAL MANYEARS: | PROFESSIONAL: | OTHER: |
|---|---|---|
| 1.5 | 1.3 | .2 |

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Studies on the properties of tubulin were continued. The reaction of the interaction of podophyllotoxin with the colchicine site was evaluated thermodynamically and with analogues. The two molecules share only the trimethoxyphenyl portion of the binding site whereas the tropolone and lignan moieties are seen by different domains. It was also shown that maytansine interacts with both vinblastine sites but the result of binding to these sites differ from those produced with vinblastine.

Membrane tubulin has been further characterized to show that the solubilized protein is competent to polymerize with soluble brain tubulin.

Project Description:

Rat brain tubulin possesses two distinct binding sites for vinblastine per molecule: a high-affinity site with an affinity constant of $6.2 \times 10^6 M^{-1}$ and a low-affinity site with an affinity constant of $8 \times 10^4 M^{-1}$. The high-affinity site is labile, with a $t_{1/2}^{37°}$ of 3.5 hr, is protected by colchicine, and is unaffected by salt, whereas the low-affinity site is stable but is inhibited by salt. Binding to both sites is rapid.

The high-affinity binding constant of vinblastine to tubulin ($6.2 \times 10^6 M^{-1}$) corresponds to the half-maximal concentration of vinblastine needed to prevent polymerization of tubulin in vitro, whereas the low-affinity binding constant ($8 \times 10^4 M^{-1}$) corresponds to the half-maximal concentration of vinblastine required to aggregate tubulin. We conclude that vinblastine binding to the high- and low-affinity sites, respectively, accounts for the depolymerization and aggregation behavior of tubulin.

The binding of [$^3$H]podophyllotoxin to tubulin, measured by a DEAE-cellulose filter paper method, occurs with an affinity constant of $1.8 \times 10^6 M^{-1}$ (37° at pH 6.7). Like colchicine, ~0.8 mol of podophyllotoxin are bound per mol of tubulin dimer, and the reaction is entropy-driven (43 cal $deg^{-1}$ $mol^{-1}$).

At 37° the association rate constant for podophyllotoxin binding is $3.8 \times 10^6 M^{-1}$, ~10 times higher than for colchicine, this is reflected in the activation energies for binding which are 14.7 kcal/mol for podophyllotoxin and 20.3 kcal/mol for colchicine. The dissociation rate, constant for the tubulin-podophyllotoxin complex is 1.9 $h^{-1}$, and the affinity constant calculated from the ratio of the rates is close to that obtained by equilibrium measurements.

Podophyllotoxin and colchicine are mutually competitive inhibitors. This can be ascribed to the fact that both compounds have a trimethoxyphenyl ring and analogues of either compound with bulky substituents in their trimethoxyphenyl moiety are unable to inhibit the binding of either of the two ligands.

Tropolone, which inhibits colchicine binding competitively, has no effect on the podophyllotoxin/tubulin reaction. Conversely, podophyllotoxin does not influence tropolone binding. Moreover, the tropolone binding site of tubulin does not show the temperature and pH lability of the colchicine and podophyllotoxin domains, hence this lability can be ascribed to the trimethoxyphenyl binding region of tubulin.

Since podophyllotoxin analogues with a modified B ring do not bind, it is concluded that both podophyllotoxin and colchicine each have at least two points of attachment to tubulin and that they share one of them, the binding region of the trimethoxyphenyl moiety.

Maytansine is a competitive inhibitor of binding for the vinca alkaloids. This effect is exerted at both the high and low affinity binding sites and occurs with a potency ($K_i$) in the range of vinblastine. Interaction with the high affinity site is accompanied by depolymerization of microtubules. However, interaction at low affinity site, while easily measured by binding studies, is not accompanied by aggregation and prevents the aggregating effect of vinblastine. Moreover, unlike vinblastine, maytansine does not protect the independent colchicine-binding site. Whether this implies that the aggregating effect of the vinca alkaloids is the result of their dimeric nature remains to be determined.

Publications:

1. Bhattacharyya, B. and Wolff, J.: Tubulin aggregation and disaggregation: mediation by two dinstinct vinblastine-binding sites. Proc. Natl. Acad. Sci. USA 73: 2375-2378, 1976.

2. Bhattacharyya, B. and Wolff, J.: Polymerisation of membrane tubulin. Nature 264: 576-577, 1976.

3. Bhattacharyya, B. and Wolff, J.: Colchicine-binding Protein Associated with Thyroid Plasma Membranes. In Robbins, J. and Braverman, L.E. (Eds): Thyroid Research. Amsterdam, Excerpta Medica, 1976, pp. 195-197

4. Cortese, F., Bhattacharyya, B. and Wolff, J.: Podophyllotoxin as a probe for the colchicine binding site of tubulin. J. Biol. Chem. 252: 1134-1140, 1977.

5. Bhattacharyya, B. and Wolff, J.: Maytansine binding to the vinblastine sites of tubulin. FEBS Letters 75: 159-162, 1977.

| SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space) | U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT** | PROJECT NUMBER Z01 AM 45017-03 CEB |
|---|---|---|

PERIOD COVERED
July 1, 1976 through September 30, 1977

TITLE OF PROJECT (80 characters or less)

The Mechanism of Action of Lithium

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: J. Wolff Medical Director CEB NIAMDD

COOPERATING UNITS (if any)

None

LAB/BRANCH
Clinical Endocrinology Branch

SECTION
Endocrine Biochemistry

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda Maryland 20014

| TOTAL MANYEARS: | PROFESSIONAL: | OTHER: |
|---|---|---|
| .2 | .1 | .1 |

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The possibility that $Li^+$ has effects on microtubule stability has been tested on brain tubulin. $Li^+$, at reasonably low concentrations, promotes polymerization and protects against depolymerizing effects of antimitotic agents.



PHS-6040
(Rev. 10-76)

Project No. Z01 AM 45017-03 CEB

Project Description:

Lithium ion, in concentrations of 0.2 - 1.0 mM, promotes tubulin polymerization, provided small concentrations of $Mg^{++}$ ion are present. This effect of lithium on polymerization, at suboptimal concentration of magnesium, is additive for the two metal ions. Both $Li^{+}$ and $Mg^{++}$ protect microtubules against the depolymerizing effects of colchicine or vinblastine.

Publications:


1. Bhattacharyya, B. and Wolff, J.: Stabilization of microtubules by lithium ion. Biochem. Biophys. Res. Commun. 73: 383-390, 1976.

2. Wolff, J.: Iodine Homeostasis. In Klein, E. and Reinwein D. (Eds.): Regulation of Thyroid Function. Stuttgard-New York, F.K. Schattauer Verlag Publishers, 1976, pp. 65-82.

3. Gershengorn, M.C., Wolff, J. and Larsen, P.R.: Thyroid-pituitary feedback during iodine repletion. J. Clin. Endocr. Metab. 43: 601-605, 1976.

| SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space) | U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT | PROJECT NUMBER Z01 AM 45018-02 CEB |
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PERIOD COVERED
July 1, 1976 through September 30, 1977

TITLE OF PROJECT (80 characters or less)
Adenylate Cyclase and Other Extracellular Products of B. pertussis: Effects on Mammalian Cells

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

| | | | |
|---|---|---|---|
| PI: | E. L. Hewlett | Clinical Associate | CEB NIAMDD |
| | J. Wolff | Medical Director | CEB NIAMDD |
| OTHER: | C. R. Manclark | Res. Microbiologist | OD DBP BB FDA |

COOPERATING UNITS (if any)
Division of Bacterial Products, Bureau of Biologics, FDA

LAB/BRANCH
Clinical Endocrinology Branch

SECTION
Endocrine Biochemistry

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20014

| TOTAL MANYEARS: | PROFESSIONAL: | OTHER: |
|---|---|---|
| 1.4 | 1.2 | .2 |

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☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Bordetella pertussis organisms produce a number of substances, some of which are biologically active in the host animal and some of which appear to be important in the metabolism of the bacteria. One component of the organism causes an increase in circulating insulin levels in the host animal. Another bacterial product, which causes dermonecrosis and death in mice and rats, was shown to be located intracellularly. The bacterial adenylate cyclase, which has been shown previously to be predominantly extracytoplasmic in this organism, demonstrates change in its activity which are dependent upon phase and conditions of growth and culture media being used. The adenylate cyclase and the factor which increases insulin levels have been shown to be present in some standard Pertussis vaccines.

Project No. Z01 AM 45018-02 CEB

Project Description:

The adenylate cyclase of Bordetella pertussis has been shown to be located predominantly in an extracellular location. The activity of this enzyme is enhanced by a component in blood agar. When the organisms are transferred to totally synthetic medium, the adenylate cyclase exhibits a rise in activity which peaks during exponential growth and subsequently declines. There is no known biological effect of this extracytoplasmic adenylate cyclase in the host animal. There is an accumulation of enzyme activity in several Pertussis vaccines tested. In addition to the adenylate cyclase, the B. pertussis organism produces several other substances. Dermonecrotic or mouse lethal toxin is a heat labile, trypsin-sensitive, protein toxin which, unlike adenylate cyclase, is intracellular and does not accumulate in the culture medium during exponential growth. This toxin may have its effect via direct transfer from attached bacteria to host cell. Another component of B. pertussis has been shown to cause elevated levels of circulating immunoreactive insulin in rats and mice. This effect on insulin levels is demonstrable in 24 hours and persists for at least 2-3 weeks. Although the mechanism is unknown, there appears to be enhanced in vitro insulin secretion by islets isolated from B. pertussis treated animals.

Publications:

1. Hewlett, E.L., Urban, M.A., Manclark, C.R. and Wolff, J.: Extracytoplasmic adenylate cyclase of Bordetella pertussis. Proc. Natl. Acad. Sci. USA 73: 1926-1930, 1976.

2. Hewlett, E.L. and Wolff, J.: Soluble adenylate cyclase from the culture medium of Bordetella pertussis: Purification and characterization. J. Bacterol. 127: 890-898, 1976.

3. Hewlett, E.L., Manclark, C.R. and Wolff, J.: Adenylate cyclase in Bordetella pertussis vaccine. J. Inf. Dis. Supplement, August, 1977.

| SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space) | U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT** | PROJECT NUMBER Z01 AM 45019-02 CEB |
|---|---|---|

PERIOD COVERED
July 1, 1976 through September 30, 1977

TITLE OF PROJECT (80 characters or less)

Peroxidase Reactions

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

| | | | |
|---|---|---|---|
| PI: | J. Pommier | Visiting Associate | CEB NIAMDD |
| | H. J. Cahnmann | Scientist Emeritus | CEB NIAMDD |
| OTHER: | J. Nunez | Director of Research | C.N.R.S. |

COOPERATING UNITS (if any)

Unité de Recherche sur la Glande Thyroide et al Régulation Hormonale, INSERM, Bicêtre, France

LAB/BRANCH
Clinical Endocrinology Branch

SECTION
Endocrine Biochemistry

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20014

| TOTAL MANYEARS: | PROFESSIONAL: | OTHER: |
|---|---|---|
| .2 | .2 | 0 |

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES XX☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Kinetic studies in solution and adsorption-elution studies using columns of agarose-DIT revealed an interrelationship between the binding of sulfhydryl compounds and of DIT to lactoperoxidase. These ligands cause a mutual enhancement of binding.



PHS-6040
(Rev. 10-76)

Project Description:

Continuation of last years investigations on the binding of thiols and of DIT to lactoperoxidase provided further insight into the interrelationships between thiol and DIT binding. The thiol shift of the Soret band of LPO is different for cysteine and for glutathione and both shifts are different from that obtained with $H_2O_2$. Consequently, they reflect binding of the thiols to LPO and are not artifacts due to a slow generation of $H_2O_2$ from cysteine or glutathione in the presence of oxygen according to: $2\ RSH + O_2 = (RS-)_2 + H_2O_2$.

Kinetic studies showed that the binding of glutathione to LPO is enhanced by DIT and that DIT is much more strongly bound to LPO in the presence of glutathione or other sulfhydryl compounds. It is possible that the binding of the two types of ligand is governed by a concerted binding mechanism.

Publications:

None

| SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space) | U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT** | PROJECT NUMBER Z01 AM 45020-02 CEB |
|---|---|---|

PERIOD COVERED

July 1, 1976 through September 30, 1977

TITLE OF PROJECT (80 characters or less)

Synthesis of Thyroxine Transport Proteins

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

| | | | |
|---|---|---|---|
| PI: | J. Goldman | Clinical Associate | CEB NIAMDD |
| | D. Glinoer | Visiting Associate | CEB NIAMDD |
| | J. Robbins | Chief, Clin. Endo. Br. | CEB NIAMDD |
| OTHER: | A. Dubois | Assistant Professor | U.S.U.H.S. |
| | R. McGuire | Research Biostatistician | VA Hosp., Wash., D.C. |
| | M. Berman | Senior Scientist | LTB NCI |

COOPERATING UNITS (if any)

Uniformed Services University of Health Sciences, Bethesda, MD; Veterans Administration Hospital, Washington, D.C.; National Cancer Institute

LAB/BRANCH

Clinical Endocrinology Branch

SECTION

Endocrine Biochemistry

INSTITUTE AND LOCATION

NIAMDD, NIH, Bethesda, Maryland 20014

| TOTAL MANYEARS: | PROFESSIONAL: | OTHER: |
|---|---|---|
| 2.6 | 1.2 | 1.4 |

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☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The effect of thyroid hormone on the synthesis and metabolism of thyroxine-binding globulin (TBG) was studied in Rhesus monkeys. The hormone caused a decrease in the distribution volume of [$^{125}$I]TBG and an increase in its disappearance rate. In contrast, there was a biphasic effect on TBG production - an increase at a physiological hormone level and a decrease at a hypothyroid level.



PHS-6040
(Rev. 10-76)

Project No. Z01 AM 45020-02 CEB

Project Description:

The effect of thyroid hormone on the synthesis and metabolism of thyroxine-binding globulin (TBG) was studied in Rhesus monkeys. Monkey [$^{125}$I]TBG was injected into 4 adult males, and serum PB$^{125}$I and Urine$^{125}$I were followed for ten days during a control period, during hyperthyroidism produced by triiodothyronine, and after thyroidectomy. TBG kinetics was analyzed by a compartmental model (SAAM 27 program). Serum TBG levels (measured by radioimmunoassay) fell transiently (30% decrease) during the induction of hyperthyroidism, returned to control levels after 2 wks, and increased by 21% after thyroidectomy. The final decay rate (k), 0.28 $\pm$ 0.01 in the control period, increased by 30% during hyperthyroidism and fell by 41% after thyroidectomy. The total distribution volume (SEV), 339 $\pm$ 45 ml, decreased by 35% during hyperthyroidism and rose by 33% after thyroidectomy. The production rate (PR), 2.4 $\pm$ 0.3 mg/d, decreased by 18% in hyperthyroidism and decreased by 22% after thyroidectomy. These results showed that thyroid hormone at normal and excess levels causes a decrease in the distribution volume of TBG, and an increase in its disappearance rate. In contrast, there is a biphasic effect on TBG production - an increase at a physiological hormone level and a decrease at a hyperthyroid level. The findings explain observed alterations of TBG levels in hyperthyroid and hypothyroid patients.

Publications:


1. Gershengorn, M. C., Glinoer, D., Fox, S. H. and Robbins, J.: Thyroxine-binding globulin synthesis by hepatocarcinoma cells in continuous culture: Effect of physiological concentrations of thyroxine. Biochem. Biophys. Res. Commun. 71: 76-82, 1976.

2. Glinoer, D., McGuire, R. A., Gershengorn, M. C., Robbins, J. and Berman, M.: Effects of estrogen on thyroxine-binding globulin metabolism in rhesus monkeys. Endocrinology 100: 9-17, 1977.

3. Glinoer, D., Gershengorn, M. C., Dubois, A. and Robbins, J.: Stimulation of thyroxine-binding globulin synthesis by isolated rhesus monkey hepatocytes after in vivo β-estradiol administration. Endocrinology 100: 807-813, 1977.

| SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space) | U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT** | PROJECT NUMBER Z01 AM 45021-01 CEB |
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PERIOD COVERED
July 1, 1976 through September 30, 1977

TITLE OF PROJECT (80 characters or less)

Pituitary Gonadotropin Pathophysiology

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

| | | | |
|---|---|---|---|
| PI: | S.W. Rosen | Medical Director | CEB NIAMDD |
| | J.M. Lieblich | Clinical Associate | CEB NIAMDD |
| OTHER: | A. Rogol | Assistant Professor | University of Virginia |
| | H. Guyda | Professor | Montreal Children's Hospital |
| | M. Schaaf | Staff Physician | Walter Reed Army Hospital |
| | J. Reardan | Staff Physician | Mercy Hospital |
| | J. Flier | Clinical Associate | DB NIAMDD |

COOPERATING UNITS (if any)
Dept. of Medicine, University of Virginia; Dept. of Pediatrics, Montreal Children's Hospital; Walter Reed Army Hospital; Dept. of Medicine, Mercy Hospital, Sacramento, CA

LAB/BRANCH
Clinical Endocrinology Branch

SECTION
Endocrine Biochemistry

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20014

| TOTAL MANYEARS: | PROFESSIONAL: | OTHER: |
|---|---|---|
| .7 | .4 | .3 |

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☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Studies continue on three syndromes of gonadotroph hypofunction: isolated, familial hypogonadotropic hypogonadism, basal encephalocele, and the syndrome of hypogonadism and anosmia (the Kallman syndrome).



PHS-6040
(Rev. 10-76)

Project Description:

A. The Syndrome of Hypogonadism and Anosmia (The Kallman Syndrome)

Studies continue on familial and episodic cases. Heterogeneity of response to LRF has been confirmed for the complete gonadotropin molecules. Radioimmunoassays have been developed for the beta subunits of both FSH and LH, and these will be applied to sera from patients with the syndrome and to obligate heterozygotes.

B. Basal Encephalocele and Hypopituitarism

Anterior pituitary hypofunction has been documented in two patients with congenital herniation of the brain through the base of the skull (trans-sphenoidal encephalocele). Case 1, a 27-year-old man, had basal LH 1.9 mIU/ml (nl 6-26) and FSH 2.4 mIU/ml (5-25), inappropriately low for the basal testosterone of 250 ng/dl (450-1100). LH and FSH samples drawn every 15 minutes for 6 hours during the day were consistently below normal. FSH and LH hyporesponded to LH-RH. GH (<2 ng/ml) was hyporesponsive to insulin hypoglycemia and arginine. Basal and provocative tests of ACTH and TSH secretion were normal. Basal levels of serum prolactin were elevated, rose normally to TRH, and fell after L-DOPA, but there was no evidence of episodic secretion during the day. Case 2, a 10-year-old boy (<3rd %ile ht, 3rd %ile wt), had GH <1 ng/ml, unresponsive to arginine , insulin hypoglycemia, or L-DOPA. He has grown on GH therapy. Basal $T_4$ (D) was 4.0 µg/dl (4.0-10.0) with undetectable TSH that hyporesponded to TRH. Prolactin hyporesponded to TRH and L-DOPA. Insulin hypoglycemia increased cortisol from 11 to 25 µg/dl. Studies are in progress on a third patient with basal encephalocele. She is 19, less than 4 ft 10 in, and has primary amenorrhea with no breast development.

These different patterns of anterior pituitary hypofunction may be due to variable disruption of hypothalamic-pituitary pathways, an associated congenital midline defect or both. Pituitary function should be evaluated in all patients with basal encephalocele and the entity should be considered in the differential diagnosis of central endocrine malfunction.

C. Familial Isolated Hypogonadotropic Hypogonadism

Three siblings with monotropic deficiency of pituitary gonadotropin have come to our attention. Two of these (1 M, 1 F) have been studied in detail. Both have normal senses of smell and normal sellar anatomy. Both also show entirely normal dynamics of growth hormone, TSH and ACTH secretion. Basal levels of serum FSH and LH, as well as response to LRF, were all low in these siblings. Low (and discordant) responses to some (but not all) stimuli of prolactin release are being examined.

Publications:

1. Goldenberg, R.L., Powell, R.D., Rosen, S.W., Marshall, J.R. and Ross, G.T.: Ovarian Morphology in Women with Anosmia and Hypogonadotropic Hypogonadism. Am. J. Obstet. Gynecol. 126: 91-94, 1976.

| SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space) | U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT** | PROJECT NUMBER Z01 AM 45022-01 CEB |
|---|---|---|

PERIOD COVERED
July 1, 1976 through September 30, 1977

TITLE OF PROJECT (80 characters or less)

Regulation of Secretion of Hypothalamic Releasing Hormones

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

| | | | |
|---|---|---|---|
| PI: | B. D. Weintraub | Senior Surgeon | CEB NIAMDD |
| OTHER: | M. Marshall | Clinical Associate | CEB NIAMDD |

COOPERATING UNITS (if any)

None

LAB/BRANCH
Clinical Endocrinology Branch

SECTION
Endocrine Biochemistry

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20014

| TOTAL MANYEARS: | PROFESSIONAL: | OTHER: |
|---|---|---|
| 1.3 | 1.1 | .2 |

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

We have developed methods to measure the hypothalamic hormones, thyrotropin releasing hormone and somatostain by radioimmunoassay, receptorassay, and bioassay techniques. These assays will be used to study the regulation of hypothalamic hormone secretion by thyroid hormones, cold stress, and various bioactive amines. Secretion of hypothalamic hormones will be correlated with the degree of their binding to receptors on dispersed pituitary cells derived from each condition.



PHS-6040

Project No. Z01 AM

Project Description:

The factors regulating the secretion of endogenous hypothalamic releasing hormones are poorly understood. We have recently developed radioimmunoassays, receptorassays, and bioassays for thyrotropin releasing hormone and are currently developing similar assays for somatostatin. The anti-TRH sera were induced by injection of animals with the haptens covalently linked to albumin and the resulting immunoassay has a sensitivity of $10^{-10}$M TRH. The receptor and bioassays employ cultures of dispersed thyrotropic tumor cells and have sensitivities of $10^{-9}$M TRH. Currently we are attempting to define the specificity of these assays by the use of various TRH analogues. Preliminary evidence suggests that there may be a disparity in the measured "TRH" among the 3 assays.

We plan to use these 3 assay systems to study the release of TRH and somatostatin by hypothalamic explants from rats during cold stress, in different states of thyroid function, and after treatment with bioactive amines. For each condition pituitary cells will be isolated and studied with regard to their TRH and somatostatin receptor-binding properties.

Publications:

None

| SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space) | U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT** | PROJECT NUMBER Z01 AM 45023-01 CEB |
|---|---|---|

PERIOD COVERED
July 1, 1976 through September 30, 1977

TITLE OF PROJECT (80 characters or less)

TSH Binding Sites: Relationship to Anti-thyroid Immunoglobulins

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

| | | |
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| OTHER : F. Pekonen | Guest Worker | CEB NIAMDD |

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None

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| TOTAL MANYEARS: | PROFESSIONAL: | OTHER: |
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☐ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Thyrotropin binding sites on intact cultured human lymphocytes have similar affinity, specificity, and salt sensitivity to those on cultured bovine or human thyroid cells but differ in the kinetics and pH characteristics of TSH binding. We are currently studying the ability of various human anti-thyroid immunoglobulins to bind to these sties and to induce a thyrotropic response.

Project No. Z01 AM

Project Description:

We have compared the properties of the thyrotropin binding sites on 2 permanent cell lines of cultured human lymphocytes to those on bovine and human thyroid cells in primary culture. The binding sites on each of the cells had a similar affinity for bovine TSH (apparent K diss. $\approx$ $10^{-7}$ to $10^{-8}$M) and displayed high specificity for TSH compared to other proteins and glycoproteins. Monovalent and divalent cations also inhibited TSH binding similarly in all the cells. However, the kinetics of TSH binding were slower in the lymphocytes than in the thyroid cells. Moreover, although both lymphocytes and thyroid cells displayed optimal TSH binding at pH 5.2, the TSH binding at pH 7.4 was considerably greater in the thyroid cells than in the lymphocytes.

It has not yet been established that these specific TSH binding sites on intact lymphocytes and thyroid cells are the relevant "receptors" at physiologic pH and salt concentrations. Nonetheless it is likely that various human anti-thyroid immunoglobulins will interact with these sites. Currently studies are in progress to determine the TSH-displacing properties of immunoglobulin concentrates derived from patients with Graves' disease, autoimmune thyroiditis, and other disease states. Ultimately the binding properties of such immunoglobulins will be correlated with other thyrotropic stimulatory effects in the same cultured cells.

Publications:

None

| SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space) | U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT** | PROJECT NUMBER Z01 AM 45024-01 CEB |
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PERIOD COVERED
July 1, 1976 through September 30, 1977

TITLE OF PROJECT (80 characters or less)

Metabolism of Thyroid Hormones

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

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| | H. J. Cahnmann | Scientist Emeritus | CEB NIAMDD |
| | J. Robbins | Chief, Clin. Endo. Br. | CEB NIAMDD |

COOPERATING UNITS (if any)

None

LAB/BRANCH
Clinical Endocrinology Branch

SECTION
Endocrine Biochemistry

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20014

| TOTAL MANYEARS: | PROFESSIONAL: | OTHER: |
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☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The metabolism of the thyroid hormones was studied in monolayer cultures of monkey hepatocarcinoma cells. The results showed the presence of a very active diodinating system for the nonphenolic ring iodines, yielding reverse triiodothyronine ($rT_3$) from thyroxine and yielding 3,5-diiodothyronine ($T_2$) and 3'-monoiodothyronine from triiodothyronine. There was a less active phenolic ring deiodinase yielding $T_2$ from $rT_3$. When the cells were starved, there was a marked enhancement of the nonphenolic ring deiodination, which probably accounts for the increased circulating levels of $rT_3$ observed in starved animals or humans.

A novel synthesis of nonphenolic ring labeled 3,5-$T_2$ was devised as a tool for further studies on iodothyronine metabolism.

Project No. Z01 AM450

Project Description:

Extrathyroidal monodeiodination of thyroxine is an important mechanism for both hormone activation and inactivation. The metabolism of the thyroid hormones was studied in monolayer cultures of monkey hepatocarcinoma cells. Triiodothyronine ($T_3$) labeled with $^{125}I$ in the phenolic ring disappeared rapidly from the medium and was converted mainly to the sulfoconjugates of 3,3'-diodothyronine ($T_2$) and 3'-monoiodothyronine ($T_1$). Little or no labeled iodide was produced. Thyroxine ($T_4$) was metabolized more slowly, producing 3,5,3'-triodothyronine or reverse triiodothyronine ($rT_3$) as well as $T_2$ sulfate, and labeled iodide. These results show that the hepatocarcinoma cells are very active in carrying out nonphenolic ring deiodination of the thyroid hormones. They should prove to be very useful in studying this biologically important pathway, which results in hormone inactivation. The cells also contain a phenolic ring deiodinase which is active in $rT_3$ metabolism.

Starvation causes a decreased conversion of thyroxine to $T_3$, and an increase in the circulating level of $rT_3$. The mechanism of the effect on $rT_3$ levels has been unknown. Brief starvation of the hepatocarcinoma cell cultures was shown to cause a marked enhancement in nonphenolic ring deiodinations resulting in the production of increased amounts of $rT_3$ as well as $T_2$ and $T_1$ from $T_4$ or $T_3$. Phenolic ring deiodination, however, was unaffected. Therefore, starvation activated only one of two separate deiodinase systems.

Further study of the nonphenolic ring deiodinase would be aided by the use of labeled 3,5-diiodothyronine ($3,5\text{-}T_2$). Availability of this compound would also permit development of a radioimmunoassay for $3,5\text{-}T_2$. A simple 2-step synthesis was devised. [$^{125}I$]diiodotyrosine is coupled with 4-hydroxy-3,5-diiodophenyl pyruvic acid to form [3,5-$^{125}I$]thyroxine. The unlabeled iodines in the phenolic ring are then removed with hydriodic acid, yielding [3,5-$^{125}I$]$T_2$.

Publications:

None

ANNUAL REPORT - 1976-1977
DIABETES BRANCH, NIAMDD

## INSULIN RECEPTORS

Insulin Receptors in Humans: Using circulating monocytes, we studied insulin receptors in people with a wide range of disorders. Recently, the abnormalities in patients with acromegaly, insulinomas, and ataxia telangiectasia have been characterized and related to their disorders of carbohydrate metabolism and to their insulin sensitivity (Bar, Muggeo). In addition, a major change in the affinity of the insulin receptor has been detected in normal subjects at 5 hours of a standard oral glucose tolerance test. This change in the receptor may relate to changes in glucose tolerance and insulin sensitivity during the 6 hours after the administration of oral glucose, but more important, indicates that modulation of the insulin receptor is part of normal physiology.

While the monocytes appear to be a reliable indicator of the acute current status of the insulin receptor, fibroblasts in culture provide a method by which the intrinsic fixed properties of the receptor can be studied in individual patients. In patients in whom the insulin binding to receptors on monocytes was disordered, studies with fibroblasts indicated that the intrinsic insulin receptors in these patients, removed from the *in vivo* situation, are in fact normal (Podskalny, Rechler, Flier, Kahn, Bar).

Growth Hormone and Glucocorticoids: Excesses and deficiencies of growth hormone and of glucocorticoids are known to be associated with major changes in carbohydrate tolerance and insulin sensitivity. Extensive studies in rodents have been completed which show that major changes occur in both concentration and affinity of receptors in these states (Kahn, Neville, Goldfine). The data generated were too complex to analyze precisely without the aid of the computer. A theoretical model of insulin binding to its receptor, which had been formulated previously, has now been converted to computer modeling which allows precise testing of each of the parameters that define the insulin binding and has allowed us to interpret complex binding data in a precise fashion (De Meyts, Kahn).

Receptors In Vivo: Previously essentially all studies of hormone binding to receptors were performed *in vitro*. A technique devised to measure insulin binding to its receptors in a quantitative fashion in the whole animals *in vivo* (Zeleznik, Roth) is now being prepared for use in people (Zeleznik, Wachslicht). These studies should commence shortly. In addition to insulin, this technique is applicable to numerous other hormones and their receptors which cannot now be studied in humans.

Receptor Evolution: To understand more clearly the nature of the insulin receptor, we have studied the insulin receptor in mammals, birds, amphibia, teleosts, and cyclostomes, with insulins from mammals, birds, teleosts and cyclostomes. In all species both natural and chemically modified insulins bound to receptors in direct proportion to their insulin-like biological potencies measured in mammals and independent of whether the insulin and receptor were from the same species. Interestingly, all natural insulins showed negative cooperativity in all receptors tested; thus negative cooperative interactions are more highly conserved than other traditional features of insulin, e.g., dimerization or $Zn^{++}$ binding. Other features of

hormone binding to its receptor also appear to be highly conserved such as the sharp pH and temperature dependence of binding. The insulin receptor functionally is more highly conserved than is the insulin molecule itself, which by most standards is functionally highly conserved (Muggeo, De Meyts, Kahn, Ginsberg).

Insulin Regulations of Receptor Affinity: We have shown previously that insulin regulates the concentration of its own receptors on cells and that this process requires not only that insulin bind to its own receptors but that the hormone-receptor complex produce other effects beyond binding. Turkey erythrocytes, which have perfectly normal insulin receptors but which lack much of the intracellular machinery of other cells, do not change their concentration of insulin receptors upon prolonged exposure to insulin but do show an increase in the affinity of the receptors (approximately 2-fold). Since proinsulin does so too in proportion to its biological potency, this effect is probably mediated via the insulin receptor itself. This quantifiable insulin response in a relatively simple cell should permit us to define more closely the nature of the insulin sensitive pathway (Kosmakos and Roth).

Compartmentalization of Hormones on Target Cells: Extensive studies of hormone interactions with their specific receptors have assumed on the basis of a limited amount of data that the hormone binds to the receptor in a fully reversible manner. Recent studies of receptor-bound insulin showed that reversibility is not complete and the degree of reversibility varies with duration of the exposure, temperature, and the type of dissociating agent used. It indicates that receptor bound insulin has been compartmentalized and these compartments are not entirely uniform. The nature of the cell bound insulin, examined by gel filtration, undergoes rapid changes. Thus, even hormone that is "specifically" bound to the receptor appears to be heterogeneous, made up of multiple components of which only part is intact insulin. These studies are being extended in the hope of finding out what the early steps in insulin action are at the target cell and natural mechanism for insulin degradation by the target cell (Kahn).

Thermodynamics of Hormone-Receptor: The theoretical model of the insulin receptor described earlier has been successfully used in computer simulation of cooperative systems. This model permits modeling of the binding system without any assumptions of a mechanistic or molecular nature and has permitted careful description and analysis of experimental data. A thermodynamic study of the insulin receptor interaction has been undertaken. The results thus far have conformed to theoretical prediction and provides the first detailed analysis of a hormone receptor interaction (De Meyts).

Macrophages and Insulin: The monocyte, a circulating macrophage, has been used widely to reflect the insulin receptors in metabolically important tissues in people. Using normal mouse spleen as well as a mouse macrophage cell line, we have studied insulin receptors on other macrophages. Physiological concentrations of insulin alter a specific immunologic function in both of these cell populations. Thus, the macrophage appears to be an insulin sensitive cell and suggests strongly that insulin directly influences function in the immune system (Bar and Koren).

## INSULIN AND GROWTH HORMONE PATIENT STUDIES

Antibodies to Insulin: The immune response to only a few pure antigens have ever been characterized. Alan Rosenthal and his colleagues in the NIAID have studied in detail the cellular and hormonal responses to many insulins and defined the immunogenetics of these responses. Recently, they had extended their study to the immune response to insulin in people. Ethically, there are limited opportunities to study well-characterized protein antigens in people. Diabetics who require insulin represent a unique opportunity for such studies. In collaboration with the Diabetes Branch of NIAMDD, the NIAID scientists have studied in detail the immune responses, both cellular and humoral, of diabetics who are having adverse immune responses to insulin therapy. These include patients who had immunological insulin resistance as well as patients with bothersome or serious allergic disorders. In addition to providing important fundamental information on the nature of the immune response, these studies form a basis for rational therapy of adverse immune reactions to insulin (Rosenthal, et al., and Kahn).

Studies of Acromegaly: We have studied patients with acromegaly in detail since 1964 and recently completed an evaluation of 47 patients who were treated with conventional supervoltage irradiation and were followed-up at 2, 5, and 10 years after treatment. The data indicate that conventional supervoltage irradiation produces a fall in growth hormone that is comparable to all other methods of therapy but with fewer side effects. Its major disadvantages appear to be the slowness of the response (about 5 years) and the time-dependent occurrence of hypopituitarism. It is not clear whether hypopituitarism is due to the radiation or a natural part of the primary disease process. The improvements in growth hormone were accompanied by improvements in the metabolic (objective) features as well as in the skeletal (subjective) problems of acromegaly (Eastman, Gorden, and Roth).

In other studies of acromegaly it has been found that thyroid disease is more common and more difficult to diagnose in patients with acromegaly (Hollander, Sober, and Roth). Hearts that are normal in size by conventional methods can be shown to be enlarged by echocardiography but heart functions appears to be unaffected (Savage, et al., and Eastman, et al.). Prolactin concentrations in acromegaly are frequently elevated. With therapy, elevated prolactin concentrations undergo a fall, while patients with normal levels of prolactin initially show a progressive rise with time (Eastman, Gorden, and Hollander). We suspect that we are observing at least two separate processes simultaneously, successful treatment of the hypersecreting tumor and the late onset of the hyperprolactinemia associated with hypopituitarism. The prevalence of diabetic complications in acromegaly (Ballantine and Gorden) and the changes in the size of the sella turcica with time are also under study. Attempts to define the etiology of acromegaly have included obtaining human pituitary tumors at surgery and to grow them in tissue culture for experimental purposes. The preliminary studies have not been successful in establishing a cell line from human pituitary tumors (Lesniak and Knazek).

Studies in Patients with Islet Cell Tumors: We have studied over the last decade patients with hypoglycemia due to insulinomas and other causes. Most recently we have studied islet cell tumors of all types in addition to insulinomas. In the present study we have shown that patients with malignant functioning islet cell tumors (but not malignant non-functioning islet cell tumors or benign tumors) have in their circulation abnormally large amounts of intact hCG or its free β-subunit or most commonly the free α-subunit. Thus, ectopic production of this hormone or of its subunits indicates metastatic spread. Previously metastases were difficult to diagnose except by their surgical demonstration. This finding is important in allowing the physician to plan a strategy of therapy since both surgical and chemotherapeutic tools are available but are imperfect. Further, the failure to find secretion of these peptides in benign disease argues against the "cell rest" theory of ectopic production of hormones and argues that ectopic production results from malignant derepression of the genome (Kahn, Weintraub, and Rosen).

Insulin-like Peptides: Previous studies had indicated that in patients with non-islet cell tumors associated with hypoglycemia, a substantial fraction have in their plasma marked elevations in the concentration of a normal insulin-like peptide, NSILA-s, without elevations of their plasma insulin. We postulated that the hypoglycemia was due to the excess levels of this insulin-like peptide. Recently, in collaboration with colleagues in Winnipeg, Canada, we showed that one patient with an adrenocortical carcinoma associated with hypoglycemia had very high levels of NSILA-s in the tumor itself and the tumor material was indistinguishable from the material found in the circulation of this patient. This is further evidence that the tumor is actually the source of the hypoglycemic agent (Megyesi, et al., and Friesen, et al.).

It has been suggested that NSILA-s, like somatomedin and other insulin-like peptides in blood, is under the control of human growth hormone. In fact, large doses of human growth hormone given intravenously as a single bolus do cause a rise in NSILA-s and in plasma somatomedins. Recently we studied a large number of situations in which growth hormone is known to be elevated and have measured simultaneously the plasma NSILA-s. In contrast to expectations, plasma NSILA-s was not elevated by any of the mechanisms by which we were able to elevate the endogenous level of hGH (Megyesi, Kahn, Gorden). These data suggest that in normal persons changes in endogenous growth hormone concentrations have no acute effect on NSILA-s. Thus, it would appear that growth hormone has an indirect chronic effect on the level of NSILA-s (and possibly other insulin-like peptides) but has no acute role in its regulation.

## OTHER STUDIES OF INSULIN-LIKE PEPTIDES AND GROWTH HORMONE

Purification: Preliminary attempts to purify NSILA-s have been undertaken taking advantage of the fact that these proteins, in contrast to all other peptide hormones, have specific binding proteins. Thus, all the low molecular weight (MW 5,000 - 10,000) components of plasma were removed by dialysis at neutral pH; the NSILA-s remained with the high molecular weight binding protein. The high molecular weight fraction

was then acidified to release the NSILA-s which was then eluted from Sephadex columns with a molecular weight of 5,000 - 10,000. This material was then purified further by electrofocusing and other sensitive techniques (Ginsberg, Kahn, Baumann).

Relaxin: Relaxin is another protein which has many structural features of the insulin molecule. This material has weak insulin-like reactivity which appears to be accounted for entirely by a small contamination with immunoreactive insulin (Havrankova and Roth); we have concluded that the relaxin molecule itself is devoid of insulin-like immunoreactivity and radioreceptor reactivity.

Multiplication Stimulating Activity: Another somatomedin-like insulin-like peptide, multiplication stimulating activity (MSA) has been characterized in further detail and purified further. MSA, somatomedin A, NSILA I and II have been characterized extensively in a wide range of culture systems and the specific receptors on these tissue types characterized in detail (Rechler, Podskalny, Nissley, et al.).

Receptors for Human Growth Hormone: We have previously shown that human growth hormone, but not other proteins, binds to specific hGH receptors and regulates the concentration of its receptors in cultured human lymphocytes of the IM-9 line. The only protein that was known to cross-react in this system was human placental lactogen with reacted with a potency of 0.3% of human growth hormone. Recent studies by others had indicated that ovine, bovine and caprine placental lactogens react as strongly with the hGH receptors as does hGH itself and we have confirmed these findings. Moreover, we have shown that hormones that were thought not to be reactive with the human growth hormone receptor do have a definite but weak reactivity. These include bovine and ovine growth hormones as well as ovine prolactin. These three proteins are about as potent as human placental lactogen. Further, all of these proteins can also cause (hormone-induced) loss of growth hormone receptors. Thus, these hormones not only bind to the receptor but probably the combination of these hormones with their specific receptors generates intracellular messengers, which are thought to be necessary for hormone-induced loss of the hormone receptor. These studies add new insights into our understanding of the relationship between pituitary growth hormones, pituitary prolactins and the placental lactogens (Lesniak and Roth).

New Approach to Receptor Assays: Previous studies have been performed to establish radioreceptor-type assays for human growth hormone, and the circulating components of human growth hormone have been characterized in this assay. In the present study, we took advantage of the fact that human growth hormone not only binds to its own receptors to occupy receptor sites but the combination of hormone with receptor causes loss of receptor sites. By combining the ability of the growth hormone to occupy its receptor and induce the loss of total receptors, we have been able to enhance the sensitivity and increase the applicability of this assay. The generalizability of this approach to establish a new principle for receptor assays now appears to be established (Eastman, Lesniak, Roth).

## ANTI-RECEPTOR ANTIBODIES

Patients with Insulin Resistant Diabetes: We have previously described a group of patients who have an unusual form of insulin resistant diabetes. We have demonstrated in their circulation antibodies that specifically bind to the insulin receptor and thereby alter its affinity or its concentration. Recently we have observed patients who have had remissions of their insulin resistant diabetes. Simultaneously, their titers of anti-receptor antibodies were reduced or disappeared entirely, and the alterations in the binding of insulin to their own receptor sites was ameliorated substantially. These studies strongly suggest an important, if not pathogenetic, role for these anti-receptor antibodies in producing the insulin resistant diabetes (Flier, Bar, Kahn).

Patient with Hypoglycemia: Other studies in patients showed one patient in whom the insulin-like property of these antibodies became the dominant one. She had intractable hypoglycemia which led to her ultimate demise. In addition, the receptors on her own cells increased several times above normal but had affinities for insulin that were markedly reduced and these receptors were not blocked by the anti-receptor antibodies. The nature of the alterations in the receptor and the mechanism for their production is unclear but appears to be of substantial interest (Flier, Bar, Gorden, Kahn).

Ataxia Telangiectasia: In addition to the group of patients with severe insulin resistant diabetes, patients with ataxia telangiectasia, a complex disorder that includes an immunological dysfunction and insulin resistant glucose intolerance, also demonstrated circulating immunoglobulins that appear to be capable of disrupting insulin binding to specific receptors.

New Method of Detection and Other Studies: Improved methods for detecting anti-receptor antibodies have been devised which detect a broader range of anti-receptor antibodies (Harrison, Flier, Kahn). The antibodies have been used further to characterize receptors. Thus, the MSA-specific receptor of human fibroblasts is blocked by the antibodies directed against the insulin receptor whereas the MSA receptor on chick fibroblasts is unaffected (Rechler). Interestingly, insulin binding sites that are present on the nuclear membrane are not blocked by the anti-receptor antibody, whereas similar receptors located on the plasma membrane are blocked (Kahn, Goldfine).

Application to Insulin Degradation: The relationship between insulin binding to its receptor and insulin degradation has been the subject of some controversy. The antibody has been used to block insulin binding to its specific receptor sites and under these conditions insulin degradation has been unaltered, again suggesting that insulin binding to its receptor is not a necessary part of insulin degradation (Flier, Kahn, Maratos-Flier).

Insulin-Like Properties of Anti-Receptor Antibodies: The characteristics of the anti-receptor antibody have been studied in detail in isolated rat adipocytes because a characteristic insulin function, stimulation of glucose oxidation, can be measured precisely. Each anti-serum not only blocked the binding of insulin to the receptor but also stimulated glucose oxidation. The ability of a given anti-serum to stimulate glucose oxidation relative to

its ability to block insulin binding varied over a wide range. Both the stimulatory and blocking activities were retained in purified immunoglobulin fractions and in pepsin fragments of IgG and both activities were lost by addition of anti-human IgG but not by anti-insulin antibodies. Preliminary experiments suggest that monovalent (Fab') fragments retain the ability to block insulin binding but lose their ability to stimulate glucose oxidation. Neuraminidase destroys both the insulin and the antibody-stimulated glucose oxidation whereas trypsin had a differential effect, destroying insulin's ability to stimulate glucose oxidation but retaining the ability of antibody to stimulate the biological response. The anti-serum also produced other characteristic effects of insulin, including lipogenesis, glycogen synthesis and stimulation of hexose transport. The anti-receptor antibody has other insulin-like characterisics in that it accelerates the dissociation of prebound insulin. Furthermore, concanavalin A, which inhibits the accelerated dissociation (negative cooperativity) induced by insulin also inhibited the accelerated dissociation of prebound insulin produced by antibody (Kahn and Baird).

| SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space) | U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT | PROJECT NUMBER Z01 AM 47,000 - 04 DB |
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PERIOD COVERED
July 1, 1976 to September 30, 1977

TITLE OF PROJECT (80 characters or less)

Cooperative Mechanisms Regulating Receptor's Affinity for Insulin

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

| | | | |
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| | E. Van Obberghen | Visiting Associate | DB, NIAMDD |
| | G. D. Knott | Computer Specialist | LSMM, DCRT |
| OTHER: | J. Roth | Medical Director | DB, NIAMDD |
| | M. Waelbroeck | Student | DB, NIAMDD |

COOPERATING UNITS (if any)
Division of Computer Research and Technology, Laboratory of Statistical and Mathematical Methodology, NIH

LAB/BRANCH
Diabetes Branch

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INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20014

| TOTAL MANYEARS: | PROFESSIONAL: | OTHER: |
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| 2.2 | 1.2 | 1.0 |

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

In this study of the negative cooperativity of the insulin receptor, we find that cytochalasin-sensitive microfilamentous structures appear to modulate the exposure of cell surface insulin receptors, while microtubules do not seem to be involved. In addition, the negative cooperativity of insulin receptors is unaltered under conditions which disrupt microtubules and microfilaments.

A theoretical model has been described previously and has been now successfully used for computer simulation of binding systems and for the analysis of experimental binding data by least-square curve fitting.

In the study of thermodynamics of the insulin-receptor interaction, the so-called "hydrophobic effect" was studied since hydrophobic reactions have a characteristic dependence on temperature. The analysis of the data indicated that the formation of the insulin-receptor complex is driven by entropy at low temperature and by enthalpy at higher temperatures. These results support the concept that the formation of the insulin-receptor complex is in large part driven by a hydrophobic effect and also provide the first detailed thermodynamic analysis of a hormone-receptor interaction.



PHS-6040

*Project Description:*

We have further studied the negatively cooperative property of the insulin receptors, i.e., the affinity of the insulin receptors is not fixed but falls with increasing occupancy. Our attention has been focused on two problems:

(a) investigating the possible role of the membrane itself and especially the participation of a cytoskeletal control (microfilaments and microtubules).

(b) designing a theoretical model that accounts for the properties of the insulin receptor and using this model for computer simulation and curve fitting of experimental data.

A. Cytosketetal Control: Since the cytoplasmic microtubules and microfilaments are involved in the mobility and distribution of surface receptors for immunoglobulins and lectins, we investigated the role of these structures in the binding of insulin and human growth hormone to their receptors on cultured human lymphocytes (IM-9 cell line).

The microfilament modifiers (cytochalasin A, B, and D) induced a decrease in the binding of insulin and hGH under steady state conditions, which was not reversed by removing the cytochalasins from the medium and was due entirely to a reduced number of receptor sites on the cell surface. The lost receptors were not detected in the medium, suggesting a redistribution within the cell. The cytochalasins failed to alter the affinity of the hormones for their receptors or the negative cooperativity of the insulin receptor. The anti-microtubule agents (vincristine, vinblastine, colchicine) had no effect on the binding of insulin and growth hormone to their receptors.

In conclusion, cytochalasin-sensitive microfilaments appear to modulate the exposure of cell surface hormone receptors, while microtubules do not seem to be involved. Our data also indicate that the negative cooperativity is unaltered by agents which interfere with the functions of microtubules and microfilaments. This observation, coupled with the finding that detergent solubilized insulin receptors still exhibit negative cooperativity with a dependency on insulin concentration superimposable to the one found with intact cells (B. H. Ginsberg, et al.), favors the hypothesis that the negative cooperativity is an intrinsic characteristic of the receptor molecule.

During this study we discovered that dimethyl sulfoxide (DMSO), which was used to solubilize the cytochalasins, inhibits the binding of insulin to its cell surface receptors. (The concentration of DMSO used in the cytochalasin experiment 0.1%, v/v did not exhibit this inhibitory effect). Thus, dimethyl sulfoxide at concentrations exceeding 0.1% (v/v) produced a dose-related inhibition of $^{125}$I-insulin binding. This inhibition was immediately present and was totally reversible. Analysis of the data for binding at steady state indicated that the decrease in binding of $^{125}$I-insulin was due to a reduced affinity of the insulin receptor without noticeable change in the concentration of receptor sites. Kinetic studies showed that the decreased affinity could be accounted for by a decreased association rate constant. At the present time the molecular mechanism underlying this inhibitory effect of dimethyl sulfoxide is not known.

*Project Description:*

B. Theoretical Modeling: The theoretical model, which accounts for the binding properties of the insulin receptor, has been described in detail in a previous report. This model has now been successfully used in computer simulation of cooperative systems. Using this approach, we are now able to describe the binding system without making any assumption of a mechanistic or molecular nature as was the case in previous approaches to the modeling of cooperative systems. This should be of advantage in the case of receptors whose nature and stoichiometry are not yet known. Furthermore, the model has not been successfully and extensively applied to the analysis of experimental data by least-square curve fitting using the MLAB program (G. D. Knott, DCRT). The binding equations derived from the previously described equation, i.e.:

$$\bar{K} = \frac{\bar{K}_e}{1 + \frac{1 - \alpha}{\alpha} \cdot \bar{Y}}$$

always involve 3 parameters that can be fitted by computer: $\bar{K}_e$, $\alpha$ and $R_o$. The model was found to fit very satisfactorily the data for insulin binding to its receptors in several systems and has been extensively used in our laboratory for the analysis of the effects of various experimental factors on $\bar{K}_e$, $\bar{K}_f$, $R_o$ and $\alpha$. ($\bar{K}_e$ = the highest affinity state of receptor; $\bar{K}_f$ = the lowest affinity state of receptor; $\alpha = \bar{K}_e/\bar{K}_f$ ; and $R_o$ = total receptor concentration).

We have initiated the study of the thermodynamics of the insulin-receptor interaction. Blundell, et al., suggested that a determinant driving force in the insulin-receptor interaction is the so-called "hydrophobic effect", that is a change in the organization of water due to removal of non-polar residues on the insulin and receptor surfaces from the medium into the hormone-receptor complex. To test this hypothesis, since hydrophobic reactions have a characteristic dependence on temperature, we have analyzed the thermodynamics of the reaction in detail. The equilibrium constant of insulin binding to the high affinity state of its receptor ($\bar{K}_e$) was measured at multiple temperatures between 5C and 37C. The van't Hoff plot of $\ln \bar{K}_e$ against 1/T was curvilinear and the free energy change $\Delta G° = -RT \ln \bar{K}_e$ also was non-linearly dependent on temperature, with a maximum near 28C. The enthalpy of the reaction, $\Delta H°$ (a measure of the heat released or absorbed) calculated from the free energy plot decreased linearly with temperature. The slope of this line, the heat capacity change, $\Delta C_p°$, was -950 cal/(mol/deg.). Such a large change in the heat capacity is indeed typical of hydrophobic reactions. The entropy change ($\Delta S°$), a measure of the degree of "disorder" in the system, also decreased linearly with temperature. The formation of the insulin-receptor complex is driven by entropy at low temperature and by enthalpy at higher temperature. These results support the concept that the formation of the insulin-receptor complex is in large part driven by a hydrophobic effect and also provide the first detailed thermodynamic analysis of a hormone-receptor interaction.

*Publications:*

1. De Meyts, P.: The Negative Cooperativity of Insulin Receptors: A Model for the Regulation of Hormone Recognition by Target Cells. *In:* Cell Membrane Receptors for Viruses, Antigens, and Antibodies, Polypeptide Hormones and Small Molecules. (Beers, R. F., and Barrett, E. G., eds.), Raven Press, New York, 1976, pp. 17 - 32.

2. De Meyts, P.: Cooperative Regulation of Hormone Binding Affinity for Cell Surface Receptors. *In:* Surface Membrane Receptors: Interface Between Cells and Environment (Bradshaw, R. A., *et al.*, eds.), Plenum Press, New York, 1976, pp. 215 - 223.

3. Simon, J., Freychet, P., Rosselin, G., and De Meyts, P.: Enhanced Binding Affinity of Chicken Insulin for Receptors in Rat Liver Membranes and Human Lymphocytes: Relationships to the Kinetic Properties of the Hormone-Receptor Interaction. *Endocrinol.*, 100, 115 - 121, 1977.

4. Van Obberghen, E., De Meyts, P., and Roth, J.: Cell Surface Receptors for Insulin and Human Growth Hormone: Effect of Microtubule and Microfilament Modifiers. *J. Biol. Chem.*, 251, 6844-6851, 1976.

5. De Meyts, P., Roth, J., Van Obberghen, E., and Waelbroeck, M.: Hormonal Control of the Affinity of Polypeptide Hormone Receptors by Negative Cooperativity and Dissociation Rate Modulation. *Endocrinology*, Proceedings of the V International Congress of Endocrinology, Hamburg, 1976, (James, V. H. T., ed.), Excerpta Medica, 1977, pp. 578 - 582, volume 1.

| SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space) | U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT** | PROJECT NUMBER ZO1 AM 47,001 - 06 DB |
|---|---|---|

PERIOD COVERED
July 1, 1976 to September 30, 1977

TITLE OF PROJECT (80 characters or less)

Regulation of Receptors on Cultured Human Lymphocytes by Insulin

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

| | | | |
|---|---|---|---|
| PI: | F. Kosmakos | Guest Worker | DB, NIAMDD |
| | U. Lang | Guest Worker | DB, NIAMDD |
| OTHER: | J. Roth | Medical Director | DB, NIAMDD |
| | D. M. Neville, Jr. | Medical Director | LNC, NIMH |

COOPERATING UNITS (if any)

Laboratory of Neurochemistry, National Institute of Mental Health

LAB/BRANCH
Diabetes Branch

SECTION

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20014

| TOTAL MANYEARS: | PROFESSIONAL: | OTHER: |
|---|---|---|
| 1.2 | 1.2 | 0 |

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

We have previously described that the concentration of insulin receptors on IM-9 lymphocytes is inversely related to the ambient insulin level. This homeostatic regulation of insulin receptor concentration by insulin is dependent upon the duration and concentration of insulin. Insulin analogue studies have shown that the loss of receptors is mediated via the receptor in direct proportion to the biological potency and affinity of the analogues. The response is temperature dependent and requires protein synthesis.

*Project Description*

Our recent studies have shown that the concentration of insulin receptors is inversely related to the concentration of ambient insulin. This regulation of the number of insulin receptors by insulin requires that the cell be metabolically active. We have investigated the ability of insulin to alter the number of insulin receptors in turkey red blood cells, which possess normal insulin receptors but which are metabolically inactive (with respect to macromolecular synthesis). The turkey red cell has insulin receptors which are normal by a variety of sensitive binding criteria; however, in this cell, intracellular organelles are sparse, the nucleus is pyknotic, and macromolecular synthesis is low or absent.

Exposure of turkey red cells to insulin ($10^{-8}$M to $10^{-6}$M) results in an increase in the level of $^{125}$I-insulin binding. The alteration in insulin binding is due to an increase in the affinity of the receptor for insulin without an apparent effect on the number of insulin receptors. The effect of insulin on receptor affinity is both time- and dose-dependent and it is reversible. In addition, insulin and proinsulin induce the alteration in receptor affinity in direct proportion to the affinity for the receptor. Thus, the insulin effects on receptor affinity appear to be a specific effect of insulin acting through the insulin receptor.

We are continuing to study the mechanism of regulation of the insulin receptor using a solubilized plasma membrane fraction.

| SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space) | U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT | PROJECT NUMBER Z01 AM 47,002 - 03 DB |
|---|---|---|

PERIOD COVERED
July 1, 1976 to September 30, 1977

TITLE OF PROJECT (80 characters or less)

Insulin Receptors in Diverse Tissues

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

| | | | |
|---|---|---|---|
| PI: | M. Muggeo | Visiting Scientist | DB, NIAMDD |
| | B. H. Ginsberg | Surgeon | DB, NIAMDD |
| OTHER: | E. Van Obberghen | Visiting Associate | DB, NIAMDD |
| | S. Falkmer | Professor | Univ. Umea, Sweden |
| | P. De Meyts | Guest Worker | DB, NIAMDD |
| | C. R. Kahn | Senior Surgeon | DB, NIAMDD |
| | J. Roth | Medical Director | DB, NIAMDD |

COOPERATING UNITS (if any)

University of Umea, Sweden

LAB/BRANCH
Diabetes Branch

SECTION

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20014

| TOTAL MANYEARS: | PROFESSIONAL: | OTHER: |
|---|---|---|
| 2.0 | 2.0 | 0 |

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☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The insulin receptor has been studied in a variety of animal species including mammals, birds, amphibians, bony fish and cyclostomes. Despite nearly a half million years of divergent evolution, the insulin receptor in all of these species maintained remarkably similar binding properties, including saturability, reversibility, specificity, temperature and pH dependence, as well as negative cooperativity. In all the species the insulin receptor binds different insulin analogues in proportion with their relative insulin-like bioactivity.



PHS-6040
(Rev. 10-76)

*Project Description:*

Previous studies from this laboratory have shown that the insulin receptor of avian erythrocytes is very similar to that of mammals. Receptors, solubilized from purified membranes of turkey erythrocytes, have been characterized and have the same binding properties as the cell-bound receptor. In the presence of insulin, the soluble receptor from a tetramer (M.W. ∿ 300,000) to a monomer (M.W. 75,000) has a molecular concomitant of the site-site interactions of the negatively cooperative type.

We have extended our studies on the evolution of the insulin receptor. We have followed at least 6 insulin binding properties in over 10 different insulin receptors from different species, including mammals, birds, amphibians, bony fish and cyclostomes. Insulin receptors from different species share some important properties including saturability, reversibility and specificity of binding of insulin. The insulin receptors show marked temperature dependence; in general, the initial binding is faster at 37° than at lower temperatures, while steady state binding is higher at the low temperatures. The steady state can be perturbated by adding cold insulin, as well as by shifting temperature. A marked pH dependence of binding has been found in all the species examined; for mammalian and avian receptors pH optimum for binding was 7.8 to 8.0. In frog and fish it was slightly more acid. In all the species examined a variety of natural and chemically modified insulins were bound to receptors in direct proportion to their insulin-like biological activity (chicken > pork > fish > proinsulin > guinea pig insulin > desasparagine-desalanine insulin). Negatively cooperative site-site interactions have been demonstrated in all of the insulin receptors not only by curvilinearity of the Scatchard plot but also by insulin induced acceleration of dissociation. In the North Atlantic hagfish, the most primitive vertebrate, we have studied both the binding properties of insulin receptors on erythrocytes and receptor binding properties of hagfish insulin. We have found that hagfish insulin was 12% as active as pork insulin on the human insulin receptor and 25% on the hagfish insulin receptor. In both receptor preparations chicken insulin was three times as active as pork insulin. The ability of hagfish insulin to induce negative cooperativity has been demonstrated and the induction was not reversed at insulin concentrations where pork insulin shows a decrease of negative cooperativity effect because of dimerization. These data are consistent with data that hagfish insulin dimers are different than mammalian insulin dimers.

Our data collected in different species of animals demonstrate that the insulin receptor has been a very stable structure over an evolutionary period of about 500 million years. In a preliminary survery of non-vertebrate species for insulin receptors, no specific binding of insulin to *Escherichia coli* or to spheroplasts from these bacteria could be demonstrated.

*Publications:*

1. Ginsberg, B. H., Kahn, C. R., and Roth, J.: The Insulin Receptor of the Turkey Erythrocyte: Purification and Characterization of the Membrane Bound Receptor. *Biochim. Biophys. Acta* 443: 227-242, 1976.

2. Ginsberg, B. H., Kahn, C. R., and Roth, J.: The Insulin Receptor of the Turkey Erythrocyte: Similarity to Mammalian Insulin Receptors. *Endocrinol.* 100: 82-90, 1977.

3. Ginsberg, B. H., Kahn, C. R., Roth, J., and De Meyts, P.: Insulin-Induced Dissociation of its Receptor into Subunits: Possible Molecular Concomitant of Negative Cooperativity. *Biochem. Biophys. Res. Commun.* 73: 1068-1074, 1976.

4. Ginsberg, B. H.: The Insulin Receptor: Properties and Regulation. *In* Litwak, G. (ed.): *Biochemical Actions of the Hormones*. New York, Academic Press, Volume 4, in press.

| SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space) | U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT** | PROJECT NUMBER Z01 AM 47,003 - 02 DB |
|---|---|---|

PERIOD COVERED
July 1, 1976 to September 30, 1977

TITLE OF PROJECT (80 characters or less)
Insulin Receptors in Fibroblasts and Their Relationship To Growth and Transformation

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

| | | | |
|---|---|---|---|
| PI: | F. C. Kosmakos | Guest Worker | DB, NIAMDD |
| | F. A. Karlsson | Guest Worker | DB, NIAMDD |
| OTHER: | J. Roth | Medical Director | DB, NIAMDD |

COOPERATING UNITS (if any)

LAB/BRANCH
Diabetes Branch

SECTION

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20014

| TOTAL MANYEARS: | PROFESSIONAL: | OTHER: |
|---|---|---|
| 2.3 | 2.2 | 0.1 |

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES XX (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Insulin receptors have been characterized in normal (Balb 3T3) and transformed mouse fibroblasts. By a variety of sensitive binding criteria, the insulin receptors on mouse fibroblasts were identical to other characterized systems.

The number of insulin receptors was low with normal growing fibroblasts and increased 2-9 fold with confluency. Transformed cells had low insulin binding, similar to normal growing cells. These changes in binding of insulin with growth were due to alterations in the number of insulin receptors, without an apparent effect on receptor affinity.

Binding of insulin to both fibroblasts and lymphocytes showed a dependence upon cell cycle. Synchronized cells showed high levels of insulin binding in $G_1$, with a drastic decrease during S and M. These results are consistent with the alterations in receptor number with growing and confluent cells.

Culturing transformed fibroblasts or human lymphocytes with various cyclic AMP analogues resulted in a 2-fold increase in the number of insulin receptors. This effect of cAMP was specific for insulin receptors, both dose- and time-dependent, and reversible. Cyclic GMP was without effect on insulin binding.

*Project Description:*

Differences in several aspects of cell membrane structure and function have been described between normal and transformed cells as well as between the exponential and stationary phases of normal cell growth. This study with the normal and transformed mouse fibroblasts was undertaken in order to permit new insight into membrane properties and to correlate hormone binding with already described transformation-dependent cell membrane functions.

Insulin receptors are well-characterized cell membrane components in several tissues, i.e., adipocytes, hepatocytes, cultured lymphocytes, etc. The characterization studies have been extended to normal (Balb 3T3) and transformed mouse fibroblasts. Insulin binds to normal fibroblasts, these receptors are similar to insulin receptors in other systems including kinetics of association, negative cooperativity, affinity, pH dependence, and specificity for insulin and insulin derivatives.

In normal fibroblasts, the binding of insulin was low in growing cells and increased 2-9 fold in confluent stationary cells. Insulin binding increased whether growth was arrested by contact inhibition or serum starvation. When serum-starved cells were stimulated to grow by the addition of fresh serum, insulin binding declined.

In cells transformed by viruses, chemicals, x-rays, or spontaneously, insulin binding was low, as in normal growing cells. In SV40-transformed cells, insulin binding increased 4-fold as cells reached higher densities in culture. The differences in binding were due to changes in the concentration of insulin receptors, without changes in affinity of the receptor for insulin. No relationship to changes in cell size was found.

Alterations in receptor number have been shown to occur with changes in cell growth. Insulin binding is low in growing cells and high in stationary, confluent cells. Since there is an alteration in the number of cells in the various stages of cell-cycle with both growth and confluency, we have investigated the effect of cell cycle on insulin binding in both fibroblasts and cultured lymphocytes. Cells were synchronized by either hydroxyurea or by the double thymidine procedure. Insulin binding was high in $G_1$ and decreased drastically during S and M phases. This was observed for both cell types and is consistent with the observation that growing cells bind less insulin than non-growing cells.

Insulin binding is low in growing normal mouse fibroblast cells and increases when growth is arrested. Transformation of cells results in a decreased level of binding, similar to that observed with growing normal cells. Since cell growth can be slowed by cyclic AMP, the effect of cyclic nucleotides on insulin receptors was investigated in SV-40-transformed fibroblasts and in a line of human lymphocytes (IM-9).

*Project Description:*

Incubation of SV40-transformed fibroblasts with a dibutyryl cyclic AMP, 8-bromo-cyclic AMP, 1-methyl-3-isobutylxanthine (MIX), a phosphodiesterase inhibitor, produced a two-fold increase in insulin receptor concentration without an effect on receptor affinity. The increase was dose-dependent, was observed after 8 hours of treatment, and reached a maximum level by 12 to 24 hours. Upon removal of the nucleotide, receptor number decreased towards basal level.

Incubation of cultured human lymphocytes (IM-9 line) with cyclic AMP derivatives or MIX also increased the number of insulin receptors without an alteration in receptor affinity. This effect was partially blocked by inhibition of protein synthesis and was independent of changes in cell cycle. The increase in insulin receptors was a specific response to cyclic AMP, as the number of receptors for human growth hormone was unaltered. Incubation with 8-bromo-cyclic GMP did not alter the level of insulin binding.

*Publications:*

1. Thomopoulos, P., Kosmakos, F. C., Pastan, I., and Lovelace, E.: Cyclic AMP Increased the Concentration of Insulin Receptors in Cultured Fibroblasts and Lymphocytes. *Biochem. Biophys. Res. Commun.*, 75, 246-252, 1977.

| SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space) | U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT** | PROJECT NUMBER Z01 AM 47,004 - 02 DB |
|---|---|---|

PERIOD COVERED
July 1, 1976 to June 30, 1977

TITLE OF PROJECT (80 characters or less)

Insulin Receptors and Insulin-Action in Macrophages

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

| | | | |
|---|---|---|---|
| PI: | R. S. Bar | Medical Director | DB, NIAMDD |
| | H. Koren | Immunologist | LI, NCI |
| OTHER: | C. Siebert | Bio Lab Tech | DB, NIAMDD |

COOPERATING UNITS (if any)

Laboratory of Immunology, National Cancer Institute

LAB/BRANCH
Diabetes Branch

SECTION

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20014

| TOTAL MANYEARS: | PROFESSIONAL: | OTHER: |
|---|---|---|
| 1.1 | 0.6 | 0.5 |

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Insulin receptors have been demonstrated on various human tissues and animal tissues. This study utilizes the insulin receptor associated with cultured mouse macrophages ($P388D_1$) and macrophage-containing cell population from normal mouse spleen. Insulin, at physiological concentrations, inhibits the ability of these cell populations to lyse antibody-coated red blood cells. Thus, these cell populations can be used to measure receptor function by following a biological response.



PHS-6040
(Rev. 10-76)

*Project Description:*

For our studies of insulin resistant conditions in people, we have used the insulin receptor on the circulating monocyte, a cell that is fundamentally a macrophage, to monitor insulin receptors elsewhere. To understand the role of the insulin receptor in macrophages, we have examined insulin receptors and insulin-mediated functions in macrophages.

In this ongoing study we have been able to demonstrate insulin receptors in a mouse macrophage cell line $P388D_1$ and in a mixed mononuclear cell preparation from normal mouse spleen. These insulin receptors are similar to receptors from other human tissue, i.e. monocytes and other mammalian cells.

Physiological concentrations of insulin alter a specific immunologic function, antibody-dependent cell-mediated cytotoxicity, in both spleen and cultured tumor cells. In spleen cells (mixed population) insulin maximally inhibits antibody-dependent cell-mediated cytotoxicity by 30% while in the more homogeneous $P388D_1$ cells, insulin inhibited antibody-dependent cell-mediated cytotoxicity by more than 70%. The mechanism by which insulin alters macrophage--mediated antibody-dependent cell-mediated cytotoxicity is unknown. The *in vivo* significance of these findings and potential role of insulin in immune function remains speculative. However, our data and data from other laboratories, suggest that insulin can alter the immune system at multiple levels, and these effects of insulin are mediated through the insulin receptor.

*Publications:*

1. Bar, R. S., Kahn, C. R., and Koren, H. S.: Insulin inhibition of antibody--dependent cytotoxicity and insulin receptors in macrophages. *Nature* **265**:632-635, 1977.

| SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space) | U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT** | PROJECT NUMBER ZO1 AM 47,005 - 05 DB |
|---|---|---|

PERIOD COVERED: July 1, 1976 to September 30, 1977

TITLE OF PROJECT (80 characters or less)

Insulin Receptors in Normal and Obese Patients

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

| | | | |
|---|---|---|---|
| PI: | R. S. Bar | Staff Fellow | DB, NIAMDD |
| | M. Muggeo | Visiting Scientist | DB, NIAMDD |
| OTHER: | C. Siebert | Bio Lab Tech | DB, NIAMDD |
| | J. Roth | Medical Director | DB, NIAMDD |
| | P. Gorden | Senior Surgeon | DB, NIAMDD |
| | C. R. Kahn | Senior Surgeon | DB, NIAMDD |
| | J. M. Podskalny | Biologist | DB, NIAMDD |
| | M. M. Rechler | Senior Surgeon | DB, NIAMDD |

COOPERATING UNITS (if any)

None

LAB/BRANCH: Diabetes Branch

SECTION:

INSTITUTE AND LOCATION: NIAMDD, NIH, Bethesda, Maryland 20014

| TOTAL MANYEARS: 2.8 | PROFESSIONAL: 2.1 | OTHER: 0.7 |
|---|---|---|

CHECK APPROPRIATE BOX(ES)

☒ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Insulin binding to circulating monocytes has been evaluated in different conditions of insulin resistance (acromegaly, ataxia-telangiectasia, hypercorticolism) as well as in patients with insulinoma and even in normal subjects. Insulin receptor concentration is inversely correlated with the concentration of plasma insulin. The affinity of receptor can be reduced by the presence of anti-receptor antibodies, or can be increased in acromegaly or changed by ingestion fo glucose in normal subjects.



PHS-6040 (Rev. 10-76)

*Project Description*

Previous studies from this laboratory have shown that insulin binding to receptors on circulating monocytes is altered in several human disease states characterized by insulin resistance, including obesity and syndromes with insulin resistance and acanthosis nigricans. The insulin defect can be accounted for either by a reduction in receptor concentration and receptor affinity or both. We have extended the binding studies on circulating monocytes to other clinical states of insulin resistance such as ataxia-telangiectasia, acromegaly and hypercortisolism. In two siblings with ataxia-telangiectasia and marked resistance to endogenous and exogenous insulin, we have found a 4 to 6-fold decrease in insulin receptor affinity. This defect has not been found in cultured fibroblasts from these patients. Whole plasma from the patients and immunoglobulin-enriched fractions of plasma inhibited the binding of insulin to receptors on lymphocytes and human placental membranes. Specific immunoprecipitation indicated that the plasma inhibitor of insulin binding was predominantly IgM in nature.

In some acromegalic patients we have found a reduction in receptor concentration which is correlated with the increased fasting plasma levels of insulin. In addition, in acromegaly, insulin receptors show an increase in affinity and this seems to be a compensatory mechanism for the decrease in concentration. Changes in receptor concentration and affinity occur also in patients with insulinomas. In these patients the concentration of insulin receptors on peripheral monocytes is inversely correlated with the plasma levels of insulin, while the receptor affinity seems to predict the timing and severity of hypoglycemia.

Changes in affinity of insulin receptors have also been observed in normal subjects 5 hours after ingestion of 100g of glucose, suggesting that insulin receptor binding also provides a mechanism for the modulation of insulin action in physiological conditions.

*Publications*

1. Bar, R., Gorden, P., Kahn, C. R., and De Meyts, P.: Fluctuations in the affinity and concentration of insulin receptors on circulating monocytes of obese patients. Effect of starvation, refeeding and dieting. *J. Clin. Invest.* **58**: 1123 -1135, 1976.

2. Bar, R. and Roth, J.: Insulin receptor status in disease states of man. *Arch. Intern. Med.* **137**: 474-481, 1977.

3. Muggeo, M., Bar, R., and Roth, J.: Change in affinity of insulin receptors following oral glucose in normal adults. *J. Clin. Endocrinol. Metab.* **44**: 1206-1209, 1977.

4. Bar, R. S., Gorden, P., Roth, J., and Siebert, C. W.: Insulin receptors in patients with insulinomas: Changes in receptor affinity and concentration. *J. Clin. Endocrinol. Metab.* 44: 1210-1213, 1977.

| SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space) | U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT | PROJECT NUMBER ZO1 AM 47,006 - 02 DB |
|---|---|---|

PERIOD COVERED
July 1, 1976 to September 30, 1977

TITLE OF PROJECT (80 characters or less)

Study of Insulin Receptors *In Vivo*

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

| | | | |
|---|---|---|---|
| PI: | A. J. Zeleznik | Guest Worker | DB, NIAMDD |
| | H. Wachslicht | Visiting Scientist | DB, NIAMDD |
| OTHER: | J. Roth | Medical Director | DB, NIAMDD |
| | M. Berman | Physicist | LPP, NCI |
| | E. McGuire | Staff Fellow | LPP, NCI |

COOPERATING UNITS (if any)

Laboratory of Theoretical Biology, National Cancer Institute

LAB/BRANCH
Diabetes Branch

SECTION

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20014

| TOTAL MANYEARS: | PROFESSIONAL: | OTHER: |
|---|---|---|
| 2.3 | 2.3 | 0 |

CHECK APPROPRIATE BOX(ES)

☒ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

We have studied the insulin receptor *in vivo* in the whole animal and developed a computer model which describes the insulin-insulin receptor interaction *in vivo*. These studies show that greater than 60% of the extrapancreatic insulin is bound to receptors during normal physiological states and that the insulin receptor *in vivo* may function as a reservoir or capacitance for the plasma hormone analogous to the plasma binding proteins for steroid and thyroid hormones.

*Project Description*

We have previously demonstrated a body compartment *in vivo* which has many properties of the insulin receptor. Thus, this compartment is specific for insulin with high receptor affinity and biopotency, and insulin equilibrates with this compartment in a saturable and reversible manner. In collaboration with Drs. Mones Berman and Elizabeth McGuire, we have developed a sophisticated computer model describing insulin binding *in vivo* in the whole animal. Results indicate that over 90% of the extrapancreatic insulin is bound to receptor during normal physiological states. In addition, as we previously postulated, insulin receptors *in vivo* may function as a reservoir or capacitance for the secreted hormone.

These studies, done in association with Dr. Helena Wachslicht, are now being applied to study the insulin receptor in humans who demonstrate pathophysiological states of insulin resistance, with the primary purpose of evaluating the concentration and affinity of insulin receptors *in vivo* in these individuals. The protocol has been approved for the human study and methods of preparation of iodolabeled insulins for administration to humans have been standardized.

| SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space) | U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT** | PROJECT NUMBER ZO1 AM 47,007 - 02 DB |
|---|---|---|

PERIOD COVERED
July 1, 1976 to September 30, 1977

TITLE OF PROJECT (80 characters or less)

Antibodies to the Insulin Receptor: Studies in Disease States and Use as a Probe of Insulin Receptor Function

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

| | | | |
|---|---|---|---|
| PI: | J. S. Flier | Surgeon | DB, NIAMDD |
| OTHER: | C. R. Kahn | Senior Surgeon | DB, NIAMDD |
| | R. S. Bar | Staff Fellow | DB, NIAMDD |
| | L. C. Harrison | Guest Worker | DB, NIAMDD |

COOPERATING UNITS (if any)

None

LAB/BRANCH
Diabetes Branch

SECTION

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20014

| TOTAL MANYEARS: | PROFESSIONAL: | OTHER: |
|---|---|---|
| 2.25 | 2.25 | 0 |

CHECK APPROPRIATE BOX(ES)

XX (a) HUMAN SUBJECTS XX (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Some patients with insulin resistant diabetes have antibodies which bind to and block their insulin receptors. In the past year, we have observed dramatic changes in the clinical and biochemical status of these patients. In two patients, improvement in their insulin resistant diabetes was accompanied by lowering in their titer of anti-receptor antibody and increased insulin binding to their cells. In one patient, insulin receptors underwent proliferation, and hypoglycemia developed; the titer of anti-receptor antibody did not change. In addition to inhibiting insulin binding, these antibodies precipitate soluble insulin receptors prepared from human placenta. Using these anti-receptor antibodies as probes, we have been able to demonstrate that most insulin degradation does not require binding to the receptor.



PHS-6040

*Project Description*

We have previously defined and characterized the mechanism of the insulin resistance which occurs in several patients with an unusual form of insulin resistant diabetes. In these patients, we have demonstrated circulating antibodies directed against their cell membrane insulin receptors and have characterized in detail the interaction of these "anti-receptor antibodies" with insulin receptors in a variety of systems.

Studies in the past year have extended our understanding of this diabetic syndrome. We have studied two patients with spontaneous remissions in their insulin resistant diabetes. During these remissions, the titer of their anti-receptor antibody was markedly reduced, and the impairment of insulin receptor binding, measured in their circulating monocytes, was significantly improved. In addition to further defining the natural history of this syndrome, these data also strongly support a pathogenetic role for these anti-receptor antibodies in the observed insulin resistance.

Another patient with this syndrome had a completely different clinical course. After several years of extremely insulin resistant diabetes and high titers of anti-receptor antibodies, she rapidly evolved into a clinical syndrome of intractable fasting hypoglycemia. At this time, anti-receptor antibodies remained at high titer, but insulin receptors, studied on peripheral monocytes, had now increased to levels several times above normal. These receptors differed from normal receptors in at least two respects: they bound insulin with low affinity and they were not blocked by anti-receptor antibodies. Such a receptor proliferation has not been previously demonstrated in any disease state.

The assay for anti-receptor antibodies which we have employed has depended upon antibody-induced inhibition of insulin binding to its receptors on a variety of tissues. We have recently established another means of detecting these antibodies. This method involves immunoprecipitation of insulin receptors solubilized from human placenta and labeled with tracer amounts of $^{125}$I-insulin. With this assay it may be possible to detect additional cases of receptor dysfunction in which antibodies might bind to a site on the receptor without immediately altering insulin binding.

Antibodies to the insulin receptor were also used as a tool to study the relationship between insulin binding and insulin degradation. This subject has been a matter of controversy recently, with some data favoring independence of binding and degradation, and other data favoring a close relationship between these two functions. We used anti-receptor antibodies to block insulin binding (to adipocytes, hepatocytes and lymphoblasts) and demonstrated that degradation of insulin by these cells was not impaired. Thus, the major fraction of insulin degradation does not require prior binding of insulin to its receptor.

*Publications*

1. Flier, J. S., Kahn, C. R., Jarrett, D. B., and Roth, J.: Characterization of antibodies to the insulin receptor: A cause of insulin resistant diabetes in man. *J. Clin. Invest.* **58**: 1442-1449, 1976.

| SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space) | U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT** | PROJECT NUMBER Z01 AM 47,008 - 07 DB |
|---|---|---|

PERIOD COVERED
July 1, 1976 to September 30, 1977

TITLE OF PROJECT (80 characters or less)

Detection and Characterization of Cell Surface Receptors Using Specific $^{125}$I-Receptor Antibodies

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

| | | | |
|---|---|---|---|
| PI: | D. B. Jarrett | Guest Worker | DB, NIAMDD |
| | E. Van Obberghen | Visiting Associate | DB, NIAMDD |
| OTHER: | J. Roth | Medical Director | BD, NIAMDD |

COOPERATING UNITS (if any)

NONE

LAB/BRANCH
Diabetes Branch

SECTION

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20014

| TOTAL MANYEARS: | PROFESSIONAL: | OTHER: |
|---|---|---|
| 1.1 | 1.1 | 0 |

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Autoantibodies directed against the insulin receptor are an uncommon cause of severe insulin resistant diabetes mellitus. Antibodies and insulin compete with each other for binding. Both insulin and antibody induce negatively cooperative site-site interactions. Concanavalin A which has previously shown to inhibit negative cooperativity induced by insulin also inhibits the accelerated dissociation of prebound antibody. The spontaneous dissociation of both prebound insulin and antibody was pH dependent, although that of insulin was more sensitive. Thus this anti-receptor autoantibody possesses extraordinary insulin-like characteristics: competing with insulin for the receptor, stimulating receptor mediated responses and inducing negative cooperativity.



PHS-6040
(Rev. 10-76)

*Project Description:*

Autoantibodies directed against the insulin receptor are an uncommon cause of severe insulin resistant diabetes mellitus. Such antibodies significantly impair receptor function by producing changes in either the available receptor number, insulin receptor affinity, or both. Furthermore, these antibodies produce biological responses through their interaction with the insulin receptor. An enriched $^{125}$I-labeled anti-receptor antibody preparation was prepared by selective cytoadsorption and elution from cells rich in insulin receptors. Direct binding studies using this labeled antibody preparation have shown that the antibody binds to cells in proportion to the insulin receptor concentration present. Exposure of cells rich in insulin receptors to some proteolytic enzymes results in a comparable reduction in the binding of both insulin and the antibody. Antibody binding was competed for only by insulin and by some plasma and IgG preparations from patients with insulin receptor autoantibodies. This competition was further studied using a double label procedure with tracer amounts of $^{131}$I-insulin and $^{125}$I-antibody. The anti-receptor autoantibodies were used to produce changes in receptor occupancy without significantly altering insulin receptor affinity, and under these conditions insulin and antibody binding were comparably reduced. Changes in insulin receptor affinity produced by low pH did not affect antibody binding even though insulin binding was severely compromised. The spontaneous dissociation of prebound insulin and antibody were pH dependent, with insulin being much more sensitive. Furthermore, the insulin induced accelerated dissociation of prebound insulin (negative cooperativity) was without effect upon the dissociation of prebound antibody. It was thus apparent that antibody binding was competitively inhibited through a decrease in the available receptor number rather than a decrease in insulin receptor affinity. While the competition for insulin binding by this antibody was predominantly due to a decrease in available insulin receptor number, at antibody concentrations which inhibited insulin binding by 80%, the antibody, like insulin, induced an accelerated dissociation of prebound insulin, but was without effect upon prebound antibody. Concanavalin A, which has previously been shown to inhibit the negative cooperativity induced by insulin also inhibited the accelerated dissociation of prebound insulin induced by this antibody. Thus this anti-receptor autoantibody possesses extraordinary insulin-like characteristics: competing with insulin at the receptor, stimulating receptor mediated responses and inducing negative cooperativity.

*Publications:*

1. Jarrett, D. B., Roth, J., Kahn, C. R., Flier. J. S.: Direct Method For Detection and Characterization of Cell Surface Receptors for Insulin by Means of $^{125}$I-labeled Autoantibodies Against the Insulin Receptor. *Proc. Natl. Acad. Sci., U.S.A.* 73: 4115-4119, 1976.

| SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space) | U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT** | PROJECT NUMBER Z01 AM 47,009 - 02 DB |
|---|---|---|

PERIOD COVERED
July 1, 1976 to September 30, 1977

TITLE OF PROJECT (80 characters or less)

Anti-Receptor Antibodies and Adipocytes

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

| | | | |
|---|---|---|---|
| PI: | C. R. Kahn | Senior Surgeon | DB, NIAMDD |
| | K. L. Baird | Bio Lab Tech | DB, NIAMDD |

COOPERATING UNITS (if any)

LAB/BRANCH
Diabetes Branch

SECTION

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20014

| TOTAL MANYEARS: | PROFESSIONAL: | OTHER: |
|---|---|---|
| 0.7 | 0.2 | 0.5 |

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS XX (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Three sera which contain anti-insulin receptor antibodies were used as probes of insulin receptor in adipocytes. All 3 blocked insulin binding. Some sera also had insulin-like biological effects. This activity resided in the $F(ab)'_2$ portion of the molecule.



PHS-6040
(Rev. 10-76)

*Project Description:*

We have previously reported a syndrome of severe insulin resistance associated with circulating antibodies directed at the insulin receptor. In this project we have used these anti-sera as probes of the insulin receptor of isolated rat adipocytes. All sera blocked $^{125}$I-insulin binding to isolated adipocytes and stimulated glucose oxidation. In each case the inhibition of binding observed at 37$^{o}$ was due to a decrease in receptor affinity with no change in receptor number. Fifty percent inhibition of binding occurred with serum dilutions of 1:5 to 1:5000. With one serum maximal stimulation of glucose oxidation occurred with only 10% inhibition of insulin binding; with the second, inhibition of binding and stimulation of glucose oxidation showed superimposable curves; while, the third inhibited insulin binding more effectively than it stimulated glucose oxidation. Cells preincubated with the latter serum in a concentration sufficient to inhibit insulin binding by 80% showed a 50-fold shift to the right in the dose response of insulin stimulated glucose oxidation. Both activities were retained in purified immunoglobulin fractions and in the F(ab)'$_2$ fragment of IgG, and were precipitated by antibodies to human IgG but not by antibodies to insulin. Interestingly, preliminary experiments suggest that the monovalent Fab fragment retains its inhibitory effect on binding, but loses its ability to stimulate glucose oxidation. When fat cells were exposed to neuraminidase, both insulin and antibody stimulated glucose oxidation decreased in parallel; by contrast, trypsin digestion had a more profound effect on insulin stimulated glucose oxidation.

In addition to effects on glucose oxidation, the highest titer antibody has other insulin-like effects. Thus, pretreatment of adipocytes with anti-receptor sera also results in a stimulation of lipogenesis, glycogen synthesis, and 2-deoxy-glucose transport.

*Publications:*

1. Kahn, C. R., Flier, J. S., Jarrett, D. B., and Roth, J.: Use of Anti-receptor Antibodies as Probes of Receptor Structure and Function. *In:* Hormonal Receptors in Digestive Tract Physiology (S. Bonfils, P. Fromageot, and G. Rosselin, eds.), North Holland Publishing Co., Amsterdam, 1977, pp. 69-78.

2. Kahn, C. R., Baird, K. L., Flier, J. S., and Jarrett, D. B.: Effects of Autoantibodies to the Insulin Receptor On Isolated Adipocytes: Studies of Insulin Binding and Action. *J. Clin. Invest.*, in press.

| SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space) | U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT** | PROJECT NUMBER Z01 AM 47,010 - 05 DB |
|---|---|---|

PERIOD COVERED
July 1, 1976 to September 30, 1977

TITLE OF PROJECT (80 characters or less)

NSILA-s and Related Peptides

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

| | | | |
|---|---|---|---|
| PI: | C. R. Kahn | Senior Surgeon | DB, NIAMDD |
| | K. Megyesi | Guest Worker | DB, NIAMDD |
| | B. Ginsberg | Surgeon | DB, NIAMDD |
| | J. Havrankova | Guest Worker | DB, NIAMDD |
| OTHER: | P. Gorden | Medical Director | DB, NIAMDD |
| | C. C. Shinn | Secretary | DB, NIAMDD |
| | D. E. Beall | Secretary | DB, NIAMDD |

COOPERATING UNITS (if any)

LAB/BRANCH
Diabetes Branch

SECTION

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20014

| TOTAL MANYEARS: | PROFESSIONAL: | OTHER: |
|---|---|---|
| 3.8 | 2.8 | 0 |

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

NSILA-s is an insulin-like peptide which reacts with its own receptor and the insulin receptor. NSILA-s is elevated in about 40% of patients with hypoglycemia and non-islet cell tumors. Extracts of the tumor contain the peptide suggesting ectopic production. NSILA-s is low in hypopituitary doses of growth hormone (GH), however, NSILA-s does not appear to respond acutely to changes in endogenous GH. Relaxin, another peptide with structural similarities to insulin, does not react with the insulin receptor or the NSILA-s receptor.



PHS-6040
(Rev. 10-76)

*Project Description:*

NSILA-s (non-suppressible insulin-like activity, soluble in acid ethanol) is a serum peptide that has insulin-like and growth-promoting activities. We have demonstrated previously that liver plasma membranes possess separate receptors for NSILA-s and insulin which overlap in their specificities for both peptides, and developed a specific and sensitive radioreceptor assay for NSILA-s in plasma. We characterized the properties and specificity of the NSILA-s receptor and compared them to the insulin receptor. Using this radioreceptor assay we have studied basal NSILA-s levels in patients with a variety of diseases. High plasma NSILA-s concentrations were observed in 30 to 50% of patients with extrapancreatic tumors and hypoglycemia. The concentrations of free NSILA-s in these plasmas (about 5% of total NSILA-s) were sufficient to produce hypoglycemia and this observation suggests an etiological role of NSILA-s in the hypoglycemia of this syndrome. Recently we also presented evidence, that in one patient with adrenocortical carcinoma and hypoglycemia the NSILA-s was produced by the tumor itself. The NSILA-s in the extract of this tumor was the same as the NSILA-s in plasma when compared by gel filtration.

Since NSILA-s and the somatomedins are similar, it has been tempting to state that these peptides may mediate growth and that both are probably under growth hormone control. The data on this point are conflicting. Plasma concentrations of NSILA-s and somatomedins are low in hypopituitary patients and in hypophysectomized animals and rise after intravenous administration of pharmacological doses of human growth hormone (hGH). Recently we have used other stimuli which result in changes of the endogenous GH concentration and studied the effect of these tests on plasma NSILA-s.

A typical dichotomy is shown by the effects of acute starvation. When a group of normal persons were fasted 48-72 hours, NSILA-s concentrations fell to almost undetectable, while growth hormone levels rose. In contrast, when obese patients were fasted 48-72 hours, NSILA-s concentrations rose, without a change in the growth hormone levels. Further, after oral glucose, growth hormone levels fell and NSILA-s levels rose. Stimuli such as hypoglycemia, glucagon, L-DOPA, chlorpromazine, estrogen and hydrocortisone produce no change or a fall in NSILA-s while growth hormone levels rise. These data suggest that in normal persons, changes in endogenous GH concentrations, at least under conditions of these tests, have no acute effect on NSILA-s.

We have also begun preliminary attempts at purification of NSILA-s related peptides taking advantage of the binding protein. Plasma is ultrafiltered at neutral pH to remove insulin and other low mol. wt. proteins and then ultrafiltered at acid pH to obtain the NSILA-s related peptides. Two peptides with different isoelectric points (6.5 and 8.3) can then be obtained from this ultrafiltrate using gel filtration and isoelectric focusing.

*Project Description:*

Another insulin-like peptide is relaxin. Relaxin is a low molecular weight peptide (∿ 6000) purified from pregnant sow ovaries causing relaxation of the pubic symphasis and dilation of the cervix. The molecular structure of relaxin resembles insulin - there are two non-identical chains probably joined by disulfide bridges, and there are amino acid structure homologies also exist between relaxin, insulin and NSILA I and II. These similarities prompted us to test two relaxin preparations in insulin radioreceptor assay. The preparation NIH-R-P1 was $10^5$ times less potent than insulin and all the activity was accounted for by a contamination by insulin according to the insulin radioimmunoassay. The more purified preparation (cma[1] from Bryant-Greenwood, University of Hawaii) did not compete with labeled insulin. These results indicate that despite structural homology insulin and relaxin are recognized by different cellular receptors.

*Publications:*

1. Megyesi, K., Kahn, C. R., Roth, J., and Gorden, P.: Physiology of Non-suppressible Insulin-like Activity. *In:* Endocrinology, Volume 2, Proceedings of the V International Congress of Endocrinology, Excerpta Medica, Amsterdam-Oxford, 1977, pp. 173-177.

2. Hyodo, T., Megyesi, K., Kahn, C. R., McLean, J. P., and Friesen, H. G.: Adrenocortical Carcinoma and Hypoglycemia: Evidence for Ectopic Production of Non-suppressible Insulin-like Activity by the Tumor. *J. Clin. Endocrinol. Metab.*, 44, 1175-1184, 1977.

3. Megyesi, K., Gorden, P., and Kahn, C. R.: Lack of a Simple Relationship Between Endogenous Growth Hormone and NSILA-s Related Peptides. *J. Clin. Endocrinol. Metab.*, in press.

| SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space) | U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT | PROJECT NUMBER Z01 AM 47,011 - 04 DB |
|---|---|---|

PERIOD COVERED
July 1, 1976 to September 30, 1977

TITLE OF PROJECT (80 characters or less)

Interaction of Multiplication Stimulating Activity (MSA) with Target Cells

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

| | | | |
|---|---|---|---|
| PI: | M. M. Rechler | Senior Surgeon | DB, NIAMDD |
| | S. P. Nissley | Senior Investigator | MB, NCI |
| OTHER: | J. M. Podskalny | Chemist | DB, NIAMDD |
| | C. R. Kahn | Senior Surgeon | DB, NIAMDD |
| | J. S. Flier | Surgeon | DB, NIAMDD |
| | D. B. Jarrett | Guest Worker | DB, NIAMDD |
| | E. R. Froesch | Professor | Univ. of Zurich |
| | J. Zapf | Professor | Univ. of Zurich |
| | L. Fryklund | Chemist | A. B. Kabi, Sweden |
| | K. Hall | Senior Physician | Karolinska Hosp., Sweden |
| | A. C. Moses | Surgeon | MB, NCI |

COOPERATING UNITS (if any)
University of Zurich, Switzerland; A. B. Kabi, Sweden; Karolinska Hospital, Sweden; Metabolism Branch, National Cancer Institute

LAB/BRANCH
Diabetes Branch

SECTION

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20014

| TOTAL MANYEARS: | PROFESSIONAL: | OTHER: |
|---|---|---|
| 2.8 | 1.8 | 1.0 |

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Multiplication stimulating activity (MSA), a 10,000 molecular weight polypeptide synthesized by a cultured rat liver cell line; exhibits remarkably similarities with somatomedins purified from human plasma -- somatomedin A, NSILA I and II -- in physical and biological properties. Extensive comparisons of these peptides in multiple specific radioreceptor assays confirms this high degree of relatedness. MSA also has overlapping reactivity with insulin. In addition, both peptides are capable of reacting with some MSA receptors and all insulin receptors. The MSA and insulin receptors of human fibroblasts appear particularly closely related, since antibodies to insulin receptors also react with MSA receptors.



PHS-6040

*Project Description*

We have extended our studies of the somatomedin-like peptide, multiplication stimulating activity (MSA), a 10,000 M.W. peptide synthesized by a line of rat liver cells in culture. In particular, we have critically examined the relationship of MSA to human somatomedins and further characterized the interaction of MSA with its receptors on target cells. In collaboration with L. Fryklund and K. Hall (Stockholm) and J. Zapf and E. R. Froesch (Zurich), we have performed a detailed comparison of MSA and homogeneous preparations of human somatomedins - somatomedin A, NSILA I and NSILA II - in an extensive series of biological and competitive binding assays. MSA showed extraordinary similarity to, but not identity with, the human somatomedins. These studies also revealed considerable diversity of cell surface receptors for somatomedin-like peptides in different cell types.

The MSA receptor of cultured human fibroblasts also shares antigenic determinants with insulin receptors on fibroblasts and other tissues but not with other MSA receptors. In studies performed in collaboration with C.R. Kahn, J. Flier, and D. B. Jarrett, autoantibodies to insulin receptors purified from a patient with acanthosis nigricans and extreme insulin resistance were found to inhibit the MSA receptors of human fibroblasts, but not the MSA receptor of chick fibroblasts or liver.

*Publications*

1. Rechler, M. M., Podskalny, J. M., and Nissley, S. P.: Interaction of multiplication-stimulating activity with chick embryo fibroblasts demonstrates a growth receptor. *Nature* **259**: 134-136, 1976.

2. Rechler, M. M., Bruni, C. B., Podskalny, J. M., and Carchman, R. A.: DNA synthesis in cultured human fibroblasts: Regulation by 3':5'-cyclic AMP. *J. Supramolec. Structure* **4**: 199-204, 1976.

3. Rechler, M. M. and Podskalny, J. M.: Insulin receptors in cultured human fibroblasts. *Diabetes* **25**: 250-255, 1976.

4. Moses, A. C., Nissley, S. P., Cohen, K. L., and Rechler, M. M.: Specific binding of a somatomedin-like polypeptide in rat serum depends on growth hormone. *Nature* **263**: 137-140, 1976.

5. Rechler, M. M., Bruni, C. B., Podskalny, J. M., Warner, W., and Carchman, R. A.: Modulation of serum-stimulated DNA synthesis in cultured human fibroblasts by cAMP. *Experimental Cell Research* **104**: 411-422, 1977.

6. Rechler, M. M., Podskalny, J. M., and Nissley, S. P.: Characterization of the binding of multiplication stimulating activity (MSA) to a receptor for growth polypeptides in chick embryo fibroblasts. *J. Biol. Chem.* **252**: 3898--3910, 1977.

7. Rechler, M. M., Nissley, S. P., Podskalny, J. M., Moses, A. C., and Fryklund, L.: Identification of a receptor for somatomedin-like polypeptides in human fibroblasts. *J. Clin. Endocrinol. Metab.* 44: 820-831, 1977.

8. Todaro, G. J., De Larco, J. E., Nissley, S. P., and Rechler, M. M.: MSA and EGF receptors in sarcoma virus transfer red cells and human fibrosarcoma cells in culture. *Nature* **267**: 526-528, 1977.

9. Nissley, S. P. and Rechler, M. M.: Multiplication stimulating activity (MSA): a somatomedin-like polypeptide from cultured rat liver cells. *National Cancer Institute Monograph* (in press).

10. Nissley, S. P., Short, P. A., Rechler, M. M., Podskalny, J. M., and Coon, H. G.: Proliferation of Buffalo rat liver cells in serum-free medium does not depend upon multiplication stimulating activity (MSA). *Cell* (in press).

11. Nissley, S. P., Rechler, M. M., Moses, A. C., Short, P. A., and Podskalny, J. M.: Proinsulin binds to a growth peptide receptor and stimulates DNA synthesis in chick embryo fibroblasts. *Endocrinology* (in press).

| SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space) | U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT** | PROJECT NUMBER ZO1 AM 47,012 - 05 DB |
|---|---|---|

PERIOD COVERED
July 1, 1976 to June 30, 1977

TITLE OF PROJECT (80 characters or less)

Regulation of Receptors on Cultured Human Lymphocytes by Growth Hormone

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

| | | | |
|---|---|---|---|
| PI: | M. A. Lesniak | Research Chemist | DB, NIAMDD |
| OTHER: | J. Roth | Medical Director | DB, NIAMDD |
| | P. Gorden | Senior Surgeon | DB, NIAMDD |

COOPERATING UNITS (if any)

None

LAB/BRANCH
Diabetes Branch

SECTION

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20014

| TOTAL MANYEARS: | PROFESSIONAL: | OTHER: |
|---|---|---|
| 1.85 | 0.35 | 0.5 |

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES XX(c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

We have previously developed a radioreceptor assay to measure human growth hormone in cultured human lymphocytes. We have also determined that hGH caused a loss in receptor concentration. We have found that non-primate growth hormones and prolactins have measurable affinities for the human growth hormone receptor which is equal to that of human placental lactogen.

Under preincubation conditions all hormones that bind to the growth hormone receptor cause a loss of receptors. Thus, we conclude that these hormones have significant intrinsic activity that is hGH-like.



PHS-6040
(Rev. 10-76)

*Project Description*

In our previous studies we have shown that hGH not only binds to its specific receptor but also regulates concentration of its own receptors in cultured IM-9 lymphocytes. We have extended these studies to related hormones. Ovine placental lactogen is as reactive as hGH with the hGH receptor on IM-9 lymphocytes which confirms the findings of Carr and Friesen with receptors of human liver. We now also show that bovine and ovine growth hormones and ovine prolactin have reactivity for the human growth hormone receptor on IM-9 lymphocutes that is of the same order of magnitude (0.03%) as that previously reported for human placental lactogen. The binding studies predict that these non-primate hormones will have biological effects on skeletal growth in primates, either as agonists or antagonists.

Previous studies have shown that when IM-9 lymphocytes are exposed to human growth hormone for 18 hours at 37°C, there is a time and concentration dependent loss of human growth hormone receptors. In the present study we show that human and ovine placental lactogens, ovine prolactin, and bovine and ovine growth hormones also produce loss of the human growth hormone receptor. Since the cellular process by which a hormone induces loss of its own receptors appears to require binding of the hormone to its receptor as well as one or more subsequent steps in hormone action, it is likely that all of the preparations that induce receptor loss will be shown to have some agonist activity of human growth hormone activity in promoting skeletal growth in primates. Further, these studies extend the interrelationships between primate and non-primate pituitary and placental hormones from what has been suggested previously from biological and structural studies.

*Publications*

1. Roth, J. and Lesniak, M. A.: *In* Cell Membrane Receptors for Viruses, Antigens and Antibodies, Polypeptide Hormones and Small Molecules (R. F. Beers, Jr., and E. G. Bassett, eds), Raven Press, New York, Regulation of human growth hormone (hGH) receptors by hGH: Studies with IM-9 lymphocytes, pp. 47-48, 1976.

2. Lesniak, M.A. and Roth, J.: Regulation of receptor concentration by homologous hormone: Effect of human growth hormone on its receptor in IM-9 lymphocytes. *J. Biol. Chem.* **251**: 3720-3729, 1976.

3. Lesniak, M. A. and Gorden, P.: *In* Hormone-Receptor Interaction - Molecular Aspects (G. S. Levey, ed), Marcel Dekker, Inc., New York. Growth hormone receptors, pp. 201-219, 1976.

4. Lesniak, M.A., Gorden, P., and Roth, J.: Reactivity of non-primate growth hormones and prolactins with human growth hormone receptors on cultured human lymphocytes. *J. Clin. Endocrinol. Metab.* **44**: 838-849, 1977.

| SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space) | U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT** | PROJECT NUMBER Z01 AM 47,013 - 05 DB |
|---|---|---|

PERIOD COVERED
July 1, 1976 to June 30, 1977

TITLE OF PROJECT (80 characters or less)

Radioreceptor Assay of Growth Hormone

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

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| | R. C. Eastman | Surgeon | DB, NIAMDD |
| | C. M. Hendricks | Bio Lab Tech | DB, NIAMDD |
| | P. Gorden | Senior Surgeon | DB, NIAMDD |

COOPERATING UNITS (if any)

None

LAB/BRANCH
Diabetes Branch

SECTION

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20014

| TOTAL MANYEARS: | PROFESSIONAL: | OTHER: |
|---|---|---|
| 2.25 | 0.75 | 1.5 |

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

In hGH radioreceptor assay unlabeled hGH competes with $^{125}$I-hGH for binding a fixed number of receptor sites on cultured human lymphocytes (IM-9). The ability of hGH to regulate its receptor concentration has been exploited to increase the sensitivity of the hGH in radioreceptor assay.

In general, radioimmunoassays are more sensitive than receptor assay than *in vivo* bioassays. The specificities of the various assays are different and these studied focus on the binding of hGH to its receptor.

*Project Description*

The binding of hormone to its specific receptor is a rapid, reversible and saturable process. Recent studies have indicated that the concentration of receptors on cells is often regulated by the ambient level of hormone to which the cells are being exposed. Thus when cultured lymphocytes of the IM-9 line, which have specific receptors for human growth hormone, are incubated for several hours in the presence of growth hormone, the total concentration of receptors falls rapidly; within a few hours a new steady state of receptor concentration is established which is maintained as long as the concentration of hormone is maintained. The new steady state concentration of receptors is inversely related to the concentration of hormone to which the cells had been exposed. Thus unlabeled hormone can reduce the binding of labeled hormone in two ways. First, by competing with the labeled hormone for occupancy of a fixed number of binding sites ("competition") and secondly by interacting with the cells to produce a decrease in the total number of receptors thereby reducing the total number of sites available for occupancy by both unlabeled and labeled hormone ("regulation").

In the present study we have exploited both of these properties of the unlabeled hormone so that a given concentration of unlabeled hormone causes a much greater reduction in the binding of $^{125}$I-labeled hormone than is achieved by competition alone. This new assay, which utilizes competition plus regulation, is more sensitive than the standard competitive binding assays, and because the structural requirements of hormone of competition and regulation may be different, additional information can be gained. It should be emphasized that the new assay is not simply a non-equilibrium binding assay, as had been reported previously in several other systems, since we obtain identical results when the unlabeled and labeled hormones are present together for 6 hours as when the unlabeled hormone is present for 6 hours, and the labeled hormone is present only for the final 90 minutes.

When hGH preparations were assayed, the correlation between the bioassay and the new assay (regulatory plus competitive) were as close as the correlation that had been noted for the bioassay and the standard (competitive) receptor assay. In the case of hGH, the receptor assays retain the specificity of the bioassay but are more precise and multi-fold more sensitive. The immunoassay, while it is much more sensitive than the receptor assay has a specificity that is somewhat different; there are several circumstances where the radioimmunoassay yields values that are both far greater or far below those obtained by receptor assays (and bioassays).

*Publications*

1. Gorden, P., Lesniak, M. A., and Eastman, R.: Evidence for higher proportion of "little" growth hormone with increased radioreceptor activity in acromegalic states. *J. Clin. Endocrinol. Metab.* **43**: 364-373, 1976.

2. Gorden, P., Lesniak, M.A., and Eastman, R. C.: Growth hormone receptors. *In* Basic Applications and Clinical Uses of Hypothalamic Hormones - Proceedings of the First International Symposium on Basic Applications and Clinical Uses of Hypothalamic Hormones, Madrid, 1975, Excerpta Medica, Amsterdam, pp. 76-86, 1976.

| SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space) | U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT** | PROJECT NUMBER Z01 AM 47,014 - 10 DB |
|---|---|---|

PERIOD COVERED
July 1, 1976 to September 30, 1977

TITLE OF PROJECT (80 characters or less)
Acromegaly

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

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| | R. C. Eastman | Surgeon | DB, NIAMDD |
| OTHER: | P. Gorden | Senior Surgeon | DB, NIAMDD |
| | C. Hollander | Professor of Medicine | New York Univ. |
| | A. Sober | Assistant Professor | Peter Bent Brigham Hosp. |
| | M. A. Lesniak | Research Chemist | DB, NIAMDD |
| | R. Knazek | Senior Surgeon | LPP, NCI |
| | D. E. Beall | Secretary | DB, NIAMDD |
| | C. C. Shinn | Secretary | DB, NIAMDD |

COOPERATING UNITS (if any) New York University (New York, NY); Peter Bent Brigham Hospital (Boston, MA); Cardiology Branch, NHLBI; Department of Radiology, Clinical Center; Laboratory of Pathophysiology, NCI; Hazelton Laboratories, Inc.

LAB/BRANCH
Diabetes Branch

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NIAMDD, NIH, Bethesda, Maryland 20014

| TOTAL MANYEARS: | PROFESSIONAL: | OTHER: |
|---|---|---|
| 2.3 | 1.0 | 1.3 |

CHECK APPROPRIATE BOX(ES)

XX (a) HUMAN SUBJECTS XX (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The standard therapy for growth hormone elevation in acromegaly has been conventional supervoltage irradiation. The data of human growth hormone levels in acromegalics who received radiotherapy has been evaluated at the ten-year level in 1976-77.

An attempt to establish a cell line from human pituitary tumors was unsuccessful.



PHS-6040
(Rev. 10-76)

*Project Description*

The ongoing project in the study of acromegaly has reached a ten-year level. Forty-seven acromegalic patients were treated with conventional supervoltabe irradiation (4000-5000 R), and were followed-up at two, five, and ten years after treatment. The mean fall in the growth hormone concentrations five years after treatment was 77% and the growth hormone (GH) was less than 10 ng/ml in 73% of the cases. In the majority, growth hormone continued to fall over the 5 to 10 year interval after treatment, so that by 10 years plasma GH was $\leq$ 10 ng/ml in 81% and $\leq$ 5 ng/ml in 69% of the patients. Also, the subjective (acral and facial changes) and the objective (metabolic) effects of growth hormone excess all showed significant improvement with time that roughly paralleled the fall in plasma growth hormone concentrations. After therapy, no patient developed extrasellar extension, and headaches improved in the majority of patients. The prevalence of hypopituitarism which was low before treatment increased progressively throughout the follow-up period. Significant loss of vision was observed in only one patient who received 5600 R and had severe systemic sarcoidosis. The only other complication was temporal hair loss. Conventional supervoltage irradiation by 5 to 10 years produces the same fall in plasma hGH as all other forms of treatment for acromegaly, including transphenoidal microsurgery. The rate of fall in plasma hGH with conventional irradiation is comparable to that of more aggressive forms of irradiation, is slower than that achieved by surgical methods but clearly has fewer serious side effects.

In addition, we have continued detailed studies of several other aspects of acromegaly. Disorders of thyroid function in acromegaly are under detailed consideration in collaboration with Dr. Charles Hollander (New York University) and Dr. Arthur Sober (Peter Bent Brigham Hospital). The evaluation of heart size and heart function in acromegaly has been carried out in cooperation with the Cardiology Branch of the National Heart, Lung and Blood Institute intramural scientists. Prolactin concentrations before and following therapy and in the absence of therapy have been studied in a large group of acromegalic patients. The changes in the size of the sella turcica during the course of treatment of acromegaly has been studied extensively in collaboration with radiologists of the Clinical Center Department of Radiology. The prevalence of diabetic complications in acromegaly are under continued study. In particular, the prevalence of retinopathy is being studied in detail in collaboration with the intramural members of the National Eye Institute.

Human pituitary tumors were obtained at surgery. The tumors were enzymatically treated, dispersed into single cell suspensions, and maintained for 6 months with medium supplemented with serum. Sera were obtained from acromegalic patients and normal volunteers. IgG from these sera were purified on DEAE cellulose columns. Aliquots of IgG concentrates or SV-40 virus were added to the dispersed human pituitary cells. Spent tissue culture medium was periodically replaced with fresh IgG concentrate in maintenance medium. The spent medium was tested for hGH of hPRL by radioimmunoassay.

Initial values of hGH secretion were in excess of 200 ng/day but gradually decreased to less than 10 ng/day. The rates of hGH secretion appeared to be unresponsive to the additions of either IgG or SV-40 virus. Rates of hPRL secretion initially ranged from 4.0 to 7.0 µg/day but decreased to 0.5 µg/day, also hPRL rate was unresponsive to additions of either IgG or SV-40 virus. Attempts to establish a human cell line from pituitary adenoma that secrete significant quantities of growth hormone have been unsuccessful (cooperating unit Project #Z01 CB 205-06 LPP).

*Appendix*

Hazelton Laboratories, Inc. are providing the radioimmunoassay service for determining prolactin and hGH concentrations in the acromegalic and normal patients as well as in the *in vitro* pituitary cell experiments. The contract purchases 0.5 person years for $5,000. Contract #263-77-C-0146CC.

| SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space) | U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT** | PROJECT NUMBER Z01 AM 47,015 - 01 DB |
|---|---|---|

PERIOD COVERED
July 1, 1976 to September 30, 1977

TITLE OF PROJECT (80 characters or less)

Clinical Studies of Insulinoma and Other Islet Cell Tumors

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

| | | | |
|---|---|---|---|
| PI: | C. R. Kahn | Senior Surgeon | DB, NIAMDD |
| | R. C. Eastman | Surgeon | DB, NIAMDD |
| | S. W. Rosen | Medical Director | CEB, NIAMDD |
| | B. D. Weintraub | Senior Surgeon | CEB, NIAMDD |
| | S. Fajans | Professor of Medicine | Univ. of Michigan |

COOPERATING UNITS (if any)

Clinical Endocrinology Branch, National Institute of Arthritis, Metabolism and Digestive Diseases; University of Michigan

LAB/BRANCH
Diabetes Branch

SECTION

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20014

| TOTAL MANYEARS: | PROFESSIONAL: | OTHER: |
|---|---|---|
| 1.0 | 1.0 | 0 |

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Ectopic secretion of hCG or its subunits has been found in 17 of 27 patients with functioning malignant islet cell tumors. In contrast, no evidence for ectopic secretion was found in 43 patients with benign tumors or 6 patients with malignant non-functional tumors. Some patients were successfully treated with streptozotocin or adriamycin.

*Project Description*

Islet cell tumors are usually recognized by clinical syndromes related to hypersecretion of one or more hormones. In general, it is possible to distinguish benign from malignant forms of this disease only on finding evidence of metastatic spread. In the present study we have evaluated 76 patients with surgically proven islet cell tumors for the possibility that ectopic secretion of hCG or its α and β subunits might be used as tumor markers. Thirty-three had malignant tumors: 14 insulinoma, 8 ZE syndrome, 3 pancreatic cholera, 1 glucagonoma, 1 ectopic ACTH and 6 non-functioning. The hCG, hCG-α and hCG-β were measured by 3 specific immunoassays and were considered elevated only if the value exceeded the highest observed in our own series of control patients. None of the 43 patients with benign disease or the 6 patients with non-functioning malignant tumors had an elevated level of hCG, hCG-α or hCG-β. In contrast, 17 of the 27 patients (63%) with functioning malignant islet cell tumors had an elevated plasma level of hCG or one of its subunits. The secretion was discordant; the most frequent finding was an elevated level of hCG-α alone. In one patient who responded to streptozotocin, the level of hCG-α fell to normal, but became abnormal again when the patient relapsed. Extracts of the tumors suggested that the markers observed in the circulation were being produced in the tumor itself. These data show that hCG and its subunits are prevalent and specific markers for islet cell carcinomas. Failure to find secretion of these peptides in benign disease argues against the "cell-rest" theory of ectopic protein production but suggests that such ectopic production results from malignant derepression of the genome.

Two patients with advanced metastatic insulinoma who failed to respond to streptozotocin were treated with adriamycin. Therapy with adriamycin (Patient #1) and adriamycin plus 5-fluorouracil (Patient #2) led to significant decreases in insulin levels or measurable disease and both patients had clinical remissions lasting 5 to 7 months. Adriamycin may be effective in patients with advanced insulinoma unresponsive to conventional chemotherapy.

*Publications*

1. Kahn, C. R., Rosen, S. W., Weintraub, B. D., Fajans, S. S., and Gorden, P.: Ectopic production of chorionic gonadotropin and its subunits by islet cell tumors: A specific marker for malignancy. *N. Engl. J. Med.*, in press.

2. Eastman, R. C., Come, S. E., Strewler, G. J., Gorden, P., and Kahn, C. R.: Adriamycin therapy for advanced insulinoma. *J. Clin. Endocrinol. Metab.* 44: 142-148, 1977.

| SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space) | U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT** | PROJECT NUMBER Z01 AM 47,016 - 01 DB |
|---|---|---|

PERIOD COVERED
July 1, 1976 to September 30, 1977

TITLE OF PROJECT (80 characters or less)
Early Steps in Insulin Action

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

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|---|---|---|---|
| PI: | C. R. Kahn | Senior Surgeon | DB, NIAMDD |
| | K. L. Baird | Bio Lab Tech | DB, NIAMDD |
| | J. T. Harmon | Guest Worker | DB, NIAMDD |

COOPERATING UNITS (if any)

LAB/BRANCH
Diabetes Branch

SECTION

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20014

| TOTAL MANYEARS: | PROFESSIONAL: | OTHER: |
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| 1.7 | 1.2 | 0.5 |

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☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The fate of insulin bound to its receptor on adipocytes was studied. The insulin appears to be rapidly compartmentalized and processed to both lower and higher molecular weight components. The role of these phenomena in insulin action is under study.

*Project Description:*

$^{125}$I-insulin binds rapidly and reversibly to its receptor on a variety of tissues. To investigate the fate of the bound insulin, adipocytes were exposed to $^{125}$I-insulin at 37C, and at selected intervals the cells were treated in an attempt to dissociate the bound insulin. By several criteria the specifically bound insulin became progressively less dissociable. Thus 2 minutes after onset of binding, 50% of the $^{125}$I-insulin was dissociated by mild acid, 65% by trypsin, and 75% by excess insulin; whereas after 60 minutes, the same treatments dissociated only 0, 35, and 50%, respectively.

To study the nature of the cell-associated radioactivity, the cell pellets were dissolved in urea, acetic acid and Triton X-100 and filtered on Sephadex G-50. Within 30 minutes 10% of the $^{125}$I-insulin bound to the cells was degraded to iodotyrosyl-fragments. This fraction increased to 35% after only 7 minutes, while the fraction of intact insulin progressively declined. A small amount of the $^{125}$I also eluted in the void volume, and this increased linearly, reaching 10% of the total by 35 minutes. The cell-associated radioactivity which was resistant to trypsin included the entire void volume material, as well as increasing fractions of the intact insulin and iodotyrosyl-peptides.

These data suggest that insulin bound to adipocytes at 37C rapidly becomes both "compartmentalized", i.e., less accessible to dissociating agents, and "processed" to both higher and lower molecular weight components. The role of these phenomena in insulin action remains uncertain.

Many experimental designs have been utilized to delineate the mechanism of hormone action. Recently, Orly and Schramm have employed a well known technique, cell fusion, to examine the interaction of the catecholamine receptor (derived from turkey erythrocytes) and the adenyl cyclase system (derived from the Friend erythroleukemia cell). These experiments have indicated that cell fusion techniques are a useful tool to examine the membrane events that couple the hormone receptor interaction to a biological response. We are now attempting to use this technique to examine the mechanism of insulin action.

ANNUAL REPORT OF THE PEDIATRIC METABOLISM BRANCH
NATIONAL INSTITUTE OF ARTHRITIS, METABOLISM AND DIGESTIVE DISEASE

In the past year investigations on cystic fibrosis (CF) have continued with special emphasis on polyamines, cyclic nucleotides and the autonomic nervous system, as well as a variety of clinical studies.

Cystic fibrosis is a genetically transmitted error of metabolism affecting primarily exocrine glands, whose secretions are either abnormal chemically or behave abnormally physio-chemically. It is the abnormal physio-chemical behavior of the "mucous secretions" which causes obstruction of organ passages and most (but by no means all) of the clinical manifestations of the disease (i.e.: pancreatic deficiency, intestinal obstruction, hepatic cirrhosis and chronic pulmonary disease), which are thus secondary and far removed from the as yet unknown basic inherited defect.

It is essential to identify and clarify this basic error of metabolism in order to devise a truly rational, effective and lasting treatment and prevention of the disease.

## I. BIOCHEMICAL AND METABOLIC STUDIES

### A. Investigations on Polyamines

We have previously shown in this laboratory an abnormal spermidine - to spermine ratio in CF homo- and heterozygotes males (Clin.Chim.Acta. 62:657, 1975) and correlated changes in females with hormonal fluctuations in the menstrual cycle (Proc. Soc. Exp. Biol.& Med., 52:81, 1976). We have also demonstrated that 90% of spermine and spermidine in whole blood in both normals and CF patients are in the erythrocytes (Blood 48:469,1976).

While the significance of this abnormality in CF is still obscure the fact that it is present in heterozygotes strongly suggests it is a gene-related phenomenon and not a secondary result of the disease. In addition, polyamines are the first well characterized metabolites of low molecular weight demonstrated to be abnormal in CF. Studies in this field are therefore being actively pursued and as part of this investigation the little known role of polyamines in normal physiology is also being investigated.

#### (a). Alterations in Blood Polyamine Levels in Pregnant and Lactating Rats - Possible Sites of Biosynthesis

Blood polyamine levels have been shown to be increased in some cancer patients as well as in the blood of CF patients. Nevertheless, very little information exists as to the chemical form, metabolic fate and function of these cations in the circulatory system. Such studies could be greatly facilitated with a readily available animal model in which blood polyamine levels change as a function of specific physiological stimuli. It has recently been reported that polyamine levels increase dramatically in mammary glands of pregnant and lactating rats. For this reason, blood polyamine levels have been examined in pregnant and lactating rats and compared to levels from aged matched virgin animals.

Blood polyamine concentrations are assessed with an amino acid analyzer. "Free" (unconjugated) polyamines are quantitated after extraction with per-

chloric acid. Conjugated polyamines are determined after hydrolyzing samples for 24 hours in equal volumes of 12N HCl and partial purification on Dowex $50^+$ columns. Aged matched virgin, pregnant and lactating rats are anesthetized with ether and bled via cardiac puncture. Ornithine decarboxylase and S-adenosyl methionine decarboxylase activities are determined by measuring the liberation of $^{14}CO_2$ from DL (1 - $^{14}C$ ornithine and S-adenosyl-L-(Carboxyl - $^{14}C$) methionine.

Blood spermidine concentrations in pregnant rats are significantly ($p < .001$) elevated by the sixth day of pregnancy when compared to values obtained from virgin rats (22.45 ± 2.30 vs 12.15 ± 1.12 nmoles/ml, respectively). Blood spermidine levels continue to increase during pregnancy reaching highest levels by the 18th day and then fall to intermediate levels observed for virgin and 18 day pregnant animals. Spermidine concentrations rise to their highest levels on the third day of lactation (42.30 ± 6.09 nmoles/ml). After this rise spermidine levels fell to virgin concentration and remained at low levels during the post-lactation period examined.

Spermidine concentrations are five times as high as spermine in rat blood. Putrescine was too low to quantitate ( < 0.5 nmoles/ml) under assay conditions. Spermine, unlike spermidine, does not increase in rat blood during pregnancy or lactation. Plasma spermidine levels were determined and found to contain less than 10% of the total whole blood spermidine levels. Blood was fractionated into leukocyte and erythrocyte-rich fractions and the amount of spermidine associated with both cell types determined. On a cell basis leukocytes contain over 160 times as much spermidine as erythrocytes. However, since there are approximately 700 erythrocytes for each leukocyte in rat blood, over 76% of whole blood spermidine is associated with erythrocytes. During pregnancy and lactation spermidine increases significantly ($p < .01$) in erythrocytes, but not in leukocytes. Plasma spermidine exists almost totally in the "free" (unconjugated) form. (Table I).

TABLE I

DISTRIBUTION OF SPERMIDINE IN LEUKOCYTES AND ERYTHROCYTES

| | nmoles/ml blood | | | |
|---|---|---|---|---|
| | FRACTIONATED BLOOD | | | UNFRACTIONATED BLOOD |
| Rat Type | WBC's | RBC's | WBC's + RBC's | WBC's + RBC's |
| V 4 | 3.95 ± 0.62 | 12.23 ± 1.06 | 16.10 ± 0.79 (76) | 12.15 ± 1.12 |
| 15 DP 3 | 4.11 ± 0.90 | 25.21 ± 4.38 | 29.32 ± 4.45 (86) | 28.29 ± 4.24 |
| 3 DL 3 | 5.39 ± 0.80 | 41.98 ± 7.19 | 47.37 ± 7.29 (89) | 42.38 ± 6.09 |

Ornithine decarboxylase, the first and possibly rate limiting enzyme in polyamine biosynthesis, increases in non-parallel fashion during pregnancy and/or lactation, suggesting that these organs are involved, at least in part, in the regulation of blood spermidine. Cellular polyamine levels and their respective biosynthetic enzymes increase dramatically during the early phase of all growth processes implicating these cations as important factors in

growth and differentiation. It therefore, comes as a surprise that the bulk of circulating spermidine is associated with erythrocytes. Perhaps erythrocytes are involved in transporting spermidine to or from actively growing tissues. Using the pregnant-lactating rat system we are initiating studies to gain a better understanding of the origin, fate, role, and regulation of circulating spermidine. (Drs. D. Lundgren and Takami Oka, NIAMDD).

(b). Putrescine Uptake in Cystic Fibrosis and Control Fibroblasts

Recently it was reported that the Vmax for putrescine uptake in CF fibroblast is increased when compared to control cells lines. If reproducible, an abnormality in CF fibroblasts would imply a direct relationship to the genetic lesion and could lead to a better understanding of the underlying cause of this genetic disease. Therefore, studies were carried out to confirm and possibly extend this observation (3).

Fibroblasts were grown in plastic microtiter wells in $CO_2$ boxes. Exponentially growing cells were washed with phosphate buffered saline (PBS), preincubated in PBS + 10 mM Tris, pH 7.4, and then incubated in PBS + 1,4 $^{14}C$ putrescine. The cells were washed three times with PBS, and then extracted with 2% sodium lauryl sulfate. The extract was added to 10 ml Aquasol and counted. All normal and CF cell lines were within two passage numbers of each other.

Putrescine uptake in both CF and control fibroblast was linear as a function of time for at least 20 min for both 24 and 48 hr cultures. Uptake as a function of substrate concentration produced hyperbolic kinetics when putrescine concentrations were varied from $10^{-6}$M to $10^{-4}$M. Protein concentrations in logarithmically growing CF fibroblast have been reported to be decreased when compared to control cells. For this reason DNA was utilized for comparison of Kinetic data (Table II). Similar results were obtained for 24 hr cultures.

TABLE II

KINETIC ANALYSIS OF PUTRESCINE UPTAKE IN 24-HOUR CULTURES OF CYSTIC FIBROSIS HOMO- AND HETEROZYGOTES AND NORMAL FIBROBLASTS

| Culture type | Maximum Velocity (pmoles/min/ µg DNA | Km µM |
|---|---|---|
| $CF_1$ | 1.4 | 4.1 |
| $CF_2$ | 2.1 | 1.7 |
| $CF_3$ | 4.1 | 2.4 |
| CF-HZ | 2.8 | 3.2 |
| $N_1$ | 0.9 | 5.2 |
| $N_2$ | 2.4 | 2.3 |
| $N_3$ | 1.2 | 3.4 |

From these results we concluded that there is no significant difference between control and CF cell lines with respect to putrescine uptake as reported by other investigators. The original investigators, after studying additional CF and control cell lines are in agreement with our conclusions. (Dr. D.W. Lundgren, NIAMDD).

## II. CYCLIC NUCLEOTIDES AND THE AUTONOMIC NERVOUS SYSTEM

The role of the cyclic nucleotides in exocrine secretory processes is still being determined, but it is suspected that they mediate the influence of the autonomic nervous system on the glands and may be essential determinants of secretion. Since cystic fibrosis is a disorder involving predominantly the exocrine glands, we have begun to study the physiology and distribution of cyclic nucleotides in CF and their response in certain tissues to $\alpha$ or $\beta$ adrenergic or cholinergic stimuli.

### 1. Isoproterenol Stimulation of cAMP in Leukocytes and Fibroblasts.

We have measured the response of intracellular cAMP to stimulation with isoproterenol and Prostaglandin $E_1$ ($PGE_1$) in mixed leukocyte preparations, granulocyte preparations, mononuclear cell preparations, and cultured skin fibroblasts. In all cell systems, basal cAMP and $PGE_1$-stimulated cAMP are normal in the CF samples, but isoproterenol-stimulated cAMP is greatly diminished in CF cells.

We have shown that this decreased response does not result from kinetic differences or differences in dose-response relations for isoproterenol. Also, it is unrelated to the degree of illness as assessed by NIH clinical score, to the drug regimens of the patients, or the presence or absence of pancreatic insufficiency. From experiments with patients with other pulmonary diseases, it does not seem to be related to lung disease per se.

We have also studied mixed leukocyte preparations from CF heterozygotes and found that isoproterenol-stimulated cAMP is intermediate between normal and CF values. Currently we are in the process of examining the abnormality further by measuring adenyl cyclase activities in broken cell preparations and measuring the number and binding affinity of $\beta$-receptors. Preliminary measures of cGMP have also been made in this system.

We have also investigated the influence of physiologic concentrations of polyamines on the cAMP-generating system in leukocytes and in our whole-cell preparations they appear to have little effect. (Dr. P.B. Davis, NIAMDD).

### 2. Plasma Norepinephrine and Dopamine - $\beta$ - hydroxylase Levels in CF.

Because of the abnormal metabolic response to isoproterenol stimulation, we studied the sympathetic nervous system by an established method. We found that CF patients had significantly lower plasma norepinephrine than age matched controls in both the standing and supine positions. Also, the expected increment in plasma dopamine - $\beta$ - hydroxylase with exercise did not occur in CF patients. (Dr. P. Davis, NIAMDD).

### 3. Cyclic AMP and Cyclic GMP in Urine

Murad et al. have reported abnormalities in cAMP and cGMP excretion in CF, compared to age-matched controls (J. Endocr. & Metab. 10:552,1975). We have determined cAMP and cGMP in 24-hour urine from adult CF patients and appropriate controls and have found that male CF patients have significantly increased urinary cAMP and cGMP. For females, however, there was such marked variability that conclusions were impossible to draw.

Because of this finding we studied urinary cAMP in three normal women around the menstrual cycle and correlated this with determinations of urinary estrogens and pregnandiol. We found significant variation of urinary cAMP around the menstrual cycle. We are currently in the process of determining cGMP in these same urine samples and of determining the day to day variation in cAMP and cGMP excreted by males.

No correlation could be determined between the NIH clinical score, a measure of severity of illness in CF, and urinary cAMP or cGMP. (Dr. P.M. Davis, NIAMDD).

These findings of decreased cAMP response to isoproterenol in both leukocytes and fibroblasts from CF homo- and heterozygotes are the first clear suggestions of a subtle defect in the autonomic nervous system or the metabolic responses to it in this disorder. The fact that many obligatory heterozygotes for the CF gene show this abnormal response would indicate it is not secondary to illness. Also, the decreased plasma norepinephrine levels are the first demonstration with modern assay techniques of catecholamine abnormalities in CF. (Dr. P. Davis, NIAMDD).

## III. CLINICAL INVESTIGATIONS

### 1. Adults with CF

As mentioned in last year's annual report CF has only recently become a clinical problem in adults because of the increased survival of patients with this disorder and because of greater awareness of its existence and therefore better diagnostic acumen. At present we are analyzing our experience with the many facets of this disease presented by 75 patients with CF between the ages of 18 and 47 years. Although the clinical syndrome in adults with CF is basically similar to that of fibrocystic children, there are important differences in the two age groups in the presenting picutre and the relative incidence of certain complications leading at times in this age group to medical and surgical emergencies, in which prompt recognition and appropriate treatment are essential. The therapeutic approach to the abdominal obstructive complications (of which 48 episodes were seen) is distinctive and requires understanding of the physio-pathology of the disease (e.g.: chronic bronchitis, and others). Main differences are in the etiology of the lung disease, pulmonary function tests, the distinctive respiratory flora, the ineffectiveness of steroids, the usual absence of bronchospasm, the poor response to assisted ventilation and conversely the absolute necessity for appropriate antibiotic therapy.

Knowledge of cystic fibrosis is unfortunately lacking among many internists and pulmonary specialists at the present time and even pediatricians often do not realize the distinctive characteristics of this disease in the older age

group. Awareness of this disorder which is the commonest cause of chronic obstructive pulmonary disease in the third decade of life and of its many intestinal, nasal, hepatic and pulmonary complications is essential to a proper diagnostic and therapeutic approach. (Drs. P. di Sant'Agnese, and P. Davis, NIAMDD).

2. Assisted Ventilation in Patients with CF

A study of results of assisted ventilation in CF patients aged 1 to 32 years was conducted with the cooperation of 9 CF Centers located in different parts of the United States. This was necessary because only a relatively small number of CF patients on assisted ventilation are seen in any one center. Indeed, it has been the impression of physicians dealing with this disorder that the results were unsatisfactory and consequently they are reluctant to commit these patients to this type of treatment. However, there is no hard data so far in the literature to support this impression.

Fifty-six episodes of assisted ventilation in 46 patients 6 months to 32 years of age were analyzed and it was found that in only 3 episodes in 3 patients the results were relatively satisfactory with a survival of 1 to 5 years after mechanical assistance. Furthermore, in two out of the three cases their backgrounds were very different from the average patient. One required a respirator because of apnea due to seizures and the other was felt to be relatively well when she developed a viral pneumonitis and at the same time infectious mononucleosis and became acutely cyanotic and hypercapneic. Conversely in 35 episodes the patients died while still on assisted ventilation which in 6 had to be extended over 100 days and up to 13 months, although the patients could never be weaned from the machine. The balance of 11 episodes was also dismal as the patients died a few days or weeks after being taken off the respirator either during the same hospitalization or survived for two months to one year's time. The lack of difference in results of various age groups was surprising; infants and young children did as poorly as older patients. Precipitating factors leading to acute respiratory failure as well as complications of assisted ventilation were identified.

Important and useful guidelines for the use of practicing pediatricians, internists, and pulmonary specialists are outlined. It will be pointed out that contrary to the experience in other types of COPD, antibiotic therapy is not only of the essence in CF, but usually sufficient to cope with the frequent relapses in bronchial obstruction which punctuate the inexorably downhill course of the pulmonary component of this disease. Assisted ventilation should not be undertaken as a last resort in patients who have reached a point where they are no longer responsive to antibiotic therapy and have severe and irreversible changes in their lungs. Disastrous results from prolonged unsuccessful ventilation and eventual death at the expense of great mental and physical suffering are almost the rule in this group. If patients belonging to this category survive their length of life is measured in only weeks or months with a very poor quality of life. Many of them are permanently ventilator-bound and will die while still on the respirator. The only indications in cystic fibrosis for assisted ventilation are: (1) When there are precipitating factors leading to an acute crisis of which several have been identified (e.g. administration of narcotics for pain; discontinuing by the patients of antibiotic therapy for a few days, etc.) (2) When the patient's level of activity has been relatively high prior to the bout of acute respiratory failure and his

condition relatively good. (3) If assisted ventilation is done preventively before performing pulmonary surgery. (Drs. P. di Sant'Agnese, and P. Davis, NIAMDD).

### 3. Glucose Intolerance in CF

On the basis of clinical observation, it was thought that patients with CF had an unusually high rate of diabetes mellitus, although none of the clinical complications characteristic of it were present, (e.g. ketoacidosis). In a clinical and pathological investigation conducted in this branch Handwerger et al. (N. Engl. J. Med. 281:451, 1969) found a variable degree of insulinopenia in 42% of CF patients with pancreatic insufficiency studied, leading to glucose intolerance and eventually to fasting hyperglycemia and glycosuria. Although not negating the possibility that "familial genetic" diabetes mellitus might occur coincidentally with CF, these authors concluded that glucose intolerance in this disease was presumably due to disruption of the islets("strangulation") by the increasingly severe pancreatic fibrosis. This insulinopenia was also found later by several other authors and more recently Stahl et al. (J. Pediat. 84:821, 1974) reported that the response of both glucagon and insulin was abnormally low after arginine challenge in 7 out of 10 patients with CF. They thus appear to confirm that increasing pancreatic fibrosis affects the secretory capacity of both $\alpha$ and $\beta$ cells, a situation comparable to that in chronic inflammatory pancreatitis. Therefore, the glucose intolerance in patients with CF is presumably entirely different from the low insulin, but high glucagon levels in "genetic" diabetes mellitus explaining the lack of complications as well as the relative ease encountered in treatment of this complication in CF.

However, from the work that has been done up to the present time it is not clear as to whether or not all or most patients with CF have insulinopenia regardless of glucose intolerance and the relation to the presence or absence of pancreatic insufficiency in such subjects has also not been clarified. Endocrine function does not seem to be primarily involved in CF, and the abnormalities reported in this and other areas are presumably secondary to the clinical and physio-pathologic effects of the disease. However, the possibility that there is a defect in the secretion of the islet cells of the pancreas and perhaps other endocrine organs has not been ruled out. Further studies to establish these relationships are being undertaken.

Accordingly CF homozygotes with and without pancreatic insufficiency are being investigated when possible including those that were studied years ago by Handwerger et al. to detect any progression in this aspect of the disease. Heterozygotes are also being investigated. Glucose tolerances and insulin and glucagon response to arginine challenge are being defined. (Drs. V. Hubbard, C. Kahn, NIAMDD, and Dr. L. Recant, VA Hospital, Washington, D.C.)

### 4. Gallstones and Bild Acid Metabolism in CF

Recent reports have shown a significant incidence of gallstones in CF patients. It has also been shown recently that untreated children with CF have a massively increased total bile acid fecal excretion, that is close to 7 times above that seen in a control population; far in excess of the slight increase seen in patients with celiac disease and most other types of malabsorption and only comparable to individuals who have had ileal resection. The mechanisms responsible for the abnormally large bile acid sequestration in CF

remains unknown. In preliminary studies of bile salt kinetics by stable isotope dilution techniques bile salt synthesis appears to be markedly elevated in untreated patients with CF and pancreatic deficiency. However, this increase may be insufficient to maintain the bile acid pool size. Administration of pancreatic enzyme supplements is apparently effective in cutting these fecal losses. However, large dosages of enzyme supplements have been reported to produce hyperuricosuria and possibly renal interstitial fibrosis; therefore indiscriminately large amounts of pancreatic enzyme supplements should not be given.

Studies have started to clarify bile acid metabolism in this disorder. Duodenal aspirates for cholesterol and bile acid determinations are being obtained. $^{14}C$ - cholic acid and $^{3}H$ - chenodoxycholic acid then are administered and blood, urine, stool, and repeat duodenal aspirates are collected to study the metabolism synthesis, reabsorption and fecal loss of bile salts in these patients. It is also planned to repeat these studies with different dosages of of pancreatic extracts in order to determine the optimal amount of pancreatin to be administered in order to avoid side effects and yet correct sufficiently the bile acid fecal loss.

It is thus hoped to clarify not only the bile acid metabolism in CF but also the significantly increased incidence of gallstones in this disorder. Many of the stones in patients with CF are cholesterol, although bilirubin stones have also been seen. Cholesterol is solubilized via incorporation into aqueous micelles of bile acid and lecithin. When cholesterol is not dissolved, the bile becomes super-saturated and cholesterol crystals are formed. These crystals accumulate around other bile substances to form gallstones. The decrease in bile acid-pool in CF might render their bowel incapable of solubilizing biliary cholesterol and induce stone formation. (Drs. P. di Sant'Agnese, V. Hubbard, NIAMDD, Dr. H. Sharp, NICHD).

## E. Other Clinical Studies

Several other clinical studies have been conducted. A survey of *Pregnancy in CF* has been conducted throughout the country and the results of 129 pregnancies in 100 females with CF are being analyzed. It is felt that pregnancy in CF is hazardous for a mother and the fetus and not advisable unless the lung disease is mild, (NIH Score > 80). Antibiotic treatment, often with anti-pseudomonas agents, is absolutely necessary during pregnancy, despite possible hazards, in order to save the mother and the offspring. At the present time no guidelines as to the best way to handle pregnancy in CF are available and these findings will have an important impact on the medical treatment of this condition.

*Syndrome of inappropriate antidiuretic hormone secretion (SIADH) in CF* was described for the first time. It may be more common than is realized in CF and is especially important to recognize promptly in this disease, as the treatment is the exact opposite of that of hyponatremia due to salt loss because of sweating, (fluid restriction, rather than administration). As both circumstances may represent a medical emergency prompt and correct diagnosis may have fateful therapeutic consequences.

With an increasing number of CF patients surviving until adulthood *hypertrophic pulmonary osteoarthropathy* is being recognized with greater frequency in our patients. Three of them have been seen with characteristic X-ray

changes but two of them are asymptomatic, although the third has a widespread and disabling disease. Interestingly this patient who is clearly symptomatic had negative X-ray findings when the diagnosis was first suspected clinically. All three patients had severe pulmonary disease and the NIH clinical score ranges between 40 and 50.

Various methods of treatment have been investigated. The effects of *Tobramycin*, an analogue of Gentamicin, were assessed and it was not found to be significantly different in the majority of patients from the latter agent. *Ticarcillin*, an analogue of carbenicillin, was also used with success in a number of patients with CF. The advantages of ticarcillin over carbenicillin, although it has the same spectrum as the latter, is that it is more potent and can be given in smaller dosages, thus decreasing the total amount of sodium administered, a factor of special importance in patients with chronic pulmonary disease and cor pulmonale. In addition, ticarcillin appears to be less hypokalemic than carbenicillin. *Azlocillin*, a new anti-pseudomonas antibiotic, is now under study. We have shown that vitamin E deficiency results in a number of subclinical abnormalities and that CF patients should receive supplements of this vitamin. The bio-availability of three different *vitamin E preparations* were studied. Absorption curves following an orally administered dose of 20 U/kg/body weight were obtained on 7 CF patients and 3 normal volunteers. Plasma tocopherol levels were determined at 0,3,6,9,12,24 and 48 hours. Maximal levels were generally observed approximately nine hours after taking the medication and the 2 water miscible alpha-tocopherol acetate preparations performed equally well indicating that the active form of the vitamin was being absorbed adequately. In contrast the fat soluble form resulted in nearly flat absorption curves in the CF patients and was clearly less well-absorbed in the normal volunteers. This study is being extended at the present time to include CF patients without pancreatic deficiency. In addition, the possibility of using this vitamin E loading test as a convenient, non-invasive diagnostic test for the eveluation of malabsorptive states other than CF is being studied. (Dr. P. di Sant'Agnese, Dr. P. Davis, Dr. V. Hubbard, NIAMDD).

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PERIOD COVERED
July 1,1976 to September 30, 1977

TITLE OF PROJECT (80 characters or less)
Biochemical and physiologic studies of polyamines

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: David W. Lundgren, Ph.D., Senior Staff Fellow, PMB:NIAMDD

COOPERATING UNITS (if any)
Takami Oka, Ph.D., LBM, NIAMDD

LAB/BRANCH
Pediatric Metabolism Branch

SECTION

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Md. 20014

| TOTAL MANYEARS: | PROFESSIONAL: | OTHER: |
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☐ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The diamine putrescine and the polyamines spermidine and spermine are ubiquitous in animal tissues. They appear to be important compounds in growth and differentiation. Although blood polyamines have been shown to be abnormal in some cancer patients as well as in individuals with cystic fibrosis and to fluctuate in the blood of women as a function of the menstrual cycle, the origin, fate, regulation, and metabolic role have not been defined. Circulating polyamine concentrations are increased in pregnant and lactating rats when compared to virgin animals. Using this animal system studies are being carried out to gain a better understanding of the physiology and biochemistry of circulating polyamines.

Objectives: We have previously shown in this laboratory an abnormal spermidine-to-spermine ratio in CF homo and heterozygotes males (Clin. Chem. Acta 62:657,1975) and correlated changes in females with hormonal fluctuations in the menstrual cycle (Proc.Soc.Exp.Biol. & Med.152:81, 1976). We have also demonstrated that 90% of spermine and spermidine in whole blood in both normals and CF patients are in the erythrocytes (Blood 48:469,1976).

While the significance of this abnormality in CF is still obscure the fact that it is present in heterozygotes strongly suggests it is a gene-related phenomenon and not a secondary result of the disease. In addition, polyamines are the first well characterized metabolites of low molecular weight demonstrated to be abnormal in CF. Studies in this field are therefore being actively pursued and as part of this investigation the little known role of polyamines in normal physiology is also being investigated.

## METHODS AND MATERIALS

### (a) Alterations in Blood Polyamine Levels in Pregnant and Lactating Rats - Possible Sites of Biosynthesis

Blood polyamines levels have been shown to be increased in some cancer patients as well as in the blood of CF patients. Nevertheless, very little information exists as to the chemical form, metabolic fate and function of these cations in the circulatory system. Such studies could be greatly facilitated with a readily available animal model in which blood polyamine levels change as a function of specific physiological stimuli. It has recently been reported that polyamine levels increase dramatically in mammary glands of pregnant and lactating rats. For this reason, blood polyamine levels have been examined in pregnant and lactating rats and compared to levels from aged matched virgin animals.

Blood polyamine concentrations are assessed with an amino acid analyzer. "Free" (unconjugated) polyamines are quantitated after extraction with perchloric acid. Conjugated polyamines were determined after hydrolyzing samples for 24 hours in equal volumes of 12N HCl and partial purification on Dowex 50 $H^+$ columns. Aged matched virgin, pregnant and lactating rats are anesthetized with ether and bled via cardiac puncture. Ornithine decarboxylase and S-adenosyl methionine decarboxylase activities are determined by measuring the liberation of $^{14}CL_2$ from DL (1 - $^{14}C$ ornithine and S-adenosyl-L-(Carboxyl - $^{14}C$) methionine.

Blood spermidine concentrations in pregnant rats are significantly ($p < .001$) elevated by the sixth day of pregnancy when compared to values obtained from virgin rats (22.45 ± 2.30 vs 12.15 ± 1.12 nmoles/ml, respectively). Blood spermidine levels continue to increase during pregnancy reaching highest levels by the 18th day and then fall to intermediate levels observed for virgin and 18 day pregnant animals. Spermidine concentrations rise to their highest levels on the third day of lactation (42.30 + 6.09 nmoles/ml). After this rise spermidine levels fell to virgin concentration and remained at low levels during the post-lactation period examined.

Spermidine concentrations are five times as high as spermine in rat blood, Putrescine was too low to quantitate ( <0.5 nmoles/ml) under our assay conditions. Spermine, unlike spermidine, does not increase in rat blood during

pregnancy or lactation. Plasma spermidine levels were determined and found to contain less than 10% of the total whole blood spermidine levels. Blood was fractionated into leukocyte and erythrocyte-rich fractions and the amount of spermidine associated with both cell types determined. On a cell basis leukocytes contain over 160 times as much spermidine as erythrocytes. However, since there are approximately 700 erythrocytes for each leukocyte in rat blood, over 76% of whole blood spermidine is associated with erythrocytes. During pregnancy and lactation spermidine increases significantly ($p < .01$) in erythrocytes but not in leukocytes. Plasma spermidine exists almost totally in the "free" (unconjugated) form. (Table I).

TABLE I

DISTRIBUTION OF SPERMIDINE IN LEUKOCYTES AND ERYTHROCYTES

| | nmoles/ml blood | | | |
|---|---|---|---|---|
| | FRACTIONATED BLOOD | | | UNFRACTIONATED BLOOD |
| RAT TYPE | WBC's | RBC's | WBC's + RBC's | WBC's + RBC's |
| V [4] | 3.95 ± 0.62 | 12.23 ± 1.06 | 16.10 ± 0.79 (76) | 12.15 ± 1.12 |
| 15 DP [3] | 4.11 ± 0.90 | 25.21 ± 4.38 | 29.32 ± 4.45 (86) | 28.29 ± 4.24 |
| 3 DL [3] | 5.39 ± 0.80 | 41.98 ± 7.19 | 47.37 ± 7.29 (89) | 42.38 ± 6.09 |

Ornithine decarboxylase, the first and possibly rate limiting enzyme in polyamine biosynthesis, increases in non-parallel fashion during pregnancy and/or lactation, suggesting that these organs are involved, at least in part, in the regulation of blood spermidine. Cellular polyamine levels and their respective biosynthetic enzymes increase dramatically during the early phase of all growth processes implicating these cations as important factors in growth and differentiation. It therefore, comes as a surprise that the bulk of circulating spermidine is associated with erythrocytes. Perhaps erythrocytes are involved in transporting spermidine to or from actively growing tissues. Using the pregnant-lactating rat system we are initiating studies to gain a better understanding of the origin, fate, role and regulation of circulating spermidine.

(b). Putrescine Uptake in Cystic Fibrosis and Control Fibroblasts

Recently it was reported that the Vmax for putrescine uptake in CF fibroblast is increased when compared to control cells lines. If reproducible an abnormality in CF fibroblast would imply a direct relationship to the genetic lesion and could lead to a better understanding of the underlying cause of this genetic disease. Therefore, studies were carried out to confirm and possibly extend this observation (3).

Fibroblasts were grown in plastic microtiter wells in $CO_2$ boxes. Exponentially growing cells were washed with phosphate buffered saline (PBS) preincubated in PBS + 10 mM Tris, pH 7.4, and then incubated in PBS + [1,4 $^{14}C$] putrescine. The cells were washed three times with PBS, and then extracted with 2% sodium lauryl sulfate. The extract was added to 10 ml Aquasol and counted.

All normal and CF cell lines were within two passage numbers of each other.

Putrescine uptake in both CF and control fibroblast was linear as a function of time for at least 20 min for both 24 and 48 hr cultures. Uptake as a function of substrate concentration produced hyperbolic kinetics when putrescine concentrations were varied from $10^{-6}$M to $10^{-4}$M. Protein concentrations in logarithmically growing CF fibroblast have been reported to be decreased when compared to control cells. For this reason DNA was utilized for comparison of kinetic data (Table II). Similar results were obtained for 24 hr cultures.

TABLE II

KINETIC ANALYSIS OF PUTRESCINE UPTAKE IN 24-HOUR CULTURES OF CYSTIC FIBROSIS HOMO- AND HETEROZYGOTES AND NORMAL FIBROBLASTS

| Culture Type | Maximum Velocity (pmoles/min/μg DNA) | Km μM |
|---|---|---|
| $CF_1$ | 1.4 | 4.1 |
| $CF_2$ | 2.1 | 1.7 |
| $CF_3$ | 4.1 | 2.4 |
| CF-HZ | 2.8 | 3.2 |
| $N_1$ | 0.9 | 5.2 |
| $N_2$ | 2.4 | 2.3 |
| $N_3$ | 1.2 | 3.4 |

From these results we concluded that there is no significant difference between control and CF cell lines with respect to putrescine uptake as reported by other investigators. The original investigators, after studying additional CF and control cell lines are in agreement with our conclusions.

Significance to Biomedical Research: The presence of an abnormal spermidine to spermine ratio in heterozygotes strongly suggests that this is a gene-related phenomenon and not a result of the disease. This polyamine is the first well characterized metabolite of low molecular weight demonstrated to be abnormal in cystic fibrosis. Its exact implications are obscure, and whether it is primary or secondary to the still unknown basic defect remains speculative. The physiology of polyamines in normal man is also being elucidated. Certainly, processes that may be abnormal in cystic fibrosis, such as glycosyl-transferase activity and salt transport, are known to be influenced by polyamines in other systems. Further work is needed to explore the relation of polyamines to pathogenesis of cystic fibrosis.

Publications:

(1) Lundgren, D.W., Hankins, J.M., Ulane, M.M., and Willison, J.W.: Putrescine uptake in cystic fibrosis fibroblasts. J. Pediatr. 90:1033-1034, 1977.

(2) Cohen, L.F., Lundgren, D.W., and Farrell, P.M., Distribution of spermidine and spermine in blood from cystic fibrosis patients and control subjects. Blood 48:469-475, 1976.

(3). Lundgren, D.W., Farrell, P.M., Cohen, L.F.: amd Hankins, J.: Fluctuations of unbound whole blood polyamine levels during the menstrual cycle. Proc. Soc. Exp. Biol. Med. 152:81-85,1976.

(4). Cohen, L.F., Farrell, P.M., Lundgren, D.W. and di Sant'Agnese, P.A.: Electrolyte values of sweat obtained by local and whole body collection methods in cystic fibrosis patients. J. Pediatr. 89:429-433, 1976.

(5). Farrell, P.M., and Lundgren,D.W.; Recent observations concerning RNA methylation and polyamine metabolism in cystic fibrosis. In Mangos. J.A. and Talamo,R.C. (Eds.): Cystic Fibrosis: Projections into the Future. New York, Stratton Intercontinental Medical Book Corp. 1976, pp223-241.

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PERIOD COVERED

July 1, 1976 to September 30, 1977

TITLE OF PROJECT (80 characters or less)

Clinical Studies in Cystic Fibrosis

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: Paul A. di Sant'Agnese, M.D., Chief, PMB:NIAMDD
Others: P.B. Davis, M.D., Ph.D., Clinical Associate, PMB, NIAMDD
V.S. Hubbard, M.D., Ph.D., Clinical Associate, PMB:NIAMDD

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SUMMARY OF WORK (200 words or less - underline keywords)

It is the long range goal of this project to study the manifestations of cystic fibrosis with particular reference to the adult age group. We are correlating our experience with 75 adult patients. We also have analyzed data regarding pregnancy in cystic fibrosis and assisted ventilation in this disease provided by cooperating CF centers across the country. We are also studying glucose intolerance and insulin and glucagon levels in cystic fibrosis to determine whether impaired α and β islet cell function in this disorder is only secondary to increasing pancreatic fibrosis or whether endocrine as well as exocrine secretion is affected in cystic fibrosis. The metabolism of bile acids in cystic fibrosis is being investigated as well as their relation to the increased incidence of gallstones in this disorder. Prevention of the massive loss of total bile acids through the stools by the administration of pancreatic extracts is being assessed. Various methods of treatment are also being investigated with special attention to newer anitbiotics and the bioavailability of different vitamin E preparations.



PHS-6040
(Rev. 10-76)

Objectives: To clarify the physio-pathology of the many clinical features of cystic fibrosis, with special reference to adults, and thus improve treatment of this condition.

## METHODS AND MATERIALS

1. Adults with CF

As mentioned in last year's annual report CF has only reeently become a clinical problem in adults because of the increased survival of patients with this disorder and because of greater awareness of its existence and therefore better diagnostic acumen. At present we are analyzing our experience with the many facets of this disease presented by 75 patients with CF between the ages of 18 and 47 years. Although the clinical syndrome in adults with CF is basically similar to that of fibrocystic children, there are important differences in the two age groups in the presenting picture and the relative incidence of certain complications leading at times in this age group to medical and surgical emergencies, in which prompt recognition and appropriate treatment are essential. The therapeutic approach to the abdominal obstructive complications (of which 48 episodes were seen) is distinctive and requires understanding of the physio-pathology of the disease. Even the pulmonary involvement in CF, despite similarities, in many respects is quite unlike that of other types of chronic obstructive pulmonary disease (e.g.: chronic bronchitis, and others). Main differences are in the etiology of the lung disease, pulmonary function tests, the distinctive respiratory flora, the ineffectiveness of steroids, the usual absence of bronchospasm, the poor response to assisted ventilation and conversely the absolute necessity for appropriate antibiotic therapy.

Knowledge of cystic fibrosis is unfortunately lacking among many internists and pulmonary specialists at the present time and even pediatricians often do not realize the distinctive characteristics of this disease in the older age group. Awareness of this disorder which is the commonest cause of chronic obstructive pulmonary disease in the third decade of life and of its many intestinal, nasal, hepatic and pulmonary complications is essential to a proper diagnostic and therapeutic approach.

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Important and useful guidelines for the use of praticing pediatricians, internists, and pulmonary specialists are outlined. It will be pointed out that contrary to the experience in other types of COPD, antibiotic therapy is not only of the essence in CF, but usually sufficient to cope with the frequent relapses in bronchial obstruction which punctuate the inexorable downhill course of the pulmonary component of this disease. Assisted ventilation should not be undertaken as a last resort in patients who have reached a point where they are no longer responsive to antibiotic therapy and have severe and irreversible changes in their lungs. Disastrous results from prolonged unsuccessful ventilation and eventual death at the expense of great mental and physical suffering are almost the rule in this group. If patients belonging to this category survive their length of life is measured in only weeks or months with a very poor quality of life. Many of them are permanently ventilator-bound and will die while still on the respirator. The only indications in cystic fibrosis for assisted ventilation are: (1) When there are precipitating factors leading to an acute crisis of which several have been identified (e.g. administration of narcotics for pain; discontinuing by the patients of antibiotic therapy for a few days, etc); (2) When the patient's level of activity has been relatively high prior to the bout of acute respiratory failure and his condition relatively good. (3) If assisted ventilation is done preventively before performing pulmonary surgery.

3. Glucose Intolerance in CF

On the basis of clinical observation, it was thought that patients with CF had an unusually high rate of diabetes mellitus, although none of the clinical complications characteristic of it were present, (e.g. ketoacidosis). In a clinical and pathological investigation conducted in this branch Handwerger et al. (N. Engl. J. Med. 281:451, 1969) found a variable degree of insulinopenia in 42% of CF patients with pancreatic insufficiency studied, leading to glucose intolerance and eventually to fasting hyperglycemia and glycosuria. Although not negating the possibility that "familial genetic" diabetes mellitus might occur coincidentally with CF, these authors concluded that glucose intolerance in this disease was presumably due to disruption of the islets ("strangulation") by the increasingly severe pancreatic fibrosis. This insulinopenia was also found later by several other authors and more recently Stahl et al. (J. Pediat. 84:821, 1974) reported that the response of both glucagon and insulin was abnormally low after arginine challenge in 7 out of 10 patients with CF. They thus appear to confirm that increasing pancreatic fibrosis affects the secretory capacity of both $\alpha$ and $\beta$ islets cells, a situation comparable to that in chronic inflammatory pancreatitis. THerefore, the glucose intolerance in patients with CF is presumably entirely different from the low insulin, but high glucagon levels in "genetic" diabetes mellitus explaining the lack of complications as well as the relative ease encountered

in treatment of this complication in CF.

However, from the work that has been done up to the present time it is not clear as to whether or not all or most patients with CF have insulinopenia whether or not they have glucose intolerance and the relation to the presence or absence of pancreatic insufficiency in such subjects has also not been clarified. Endocrine function does not seem to be primarily involved in CF, and the abnormalities reported in this and other areas are presumably secondary to the clinical and physio-pathologic effects of the disease. However, the possibility that there is a basic defect in the secretion of the islet cells of the pancreas and perhaps other endocrine organs has not been ruled out. Further studies to establish these relationships are being undertaken.

Accordingly CF homozygotes with and without pancreatic insufficiency are being studied when possible including those that were studied years ago by Handwerger et al. to detect any progression in this aspect of the disease. Heterozygotes are also being investigated. Glucose tolerances and insulin and glucagon response to arginine challenge are being defined.

4. Gallstones and Bile Acids Metabolism in CF

Recent reports have shown a significant incidence of gallstones in CF patients. It has also been shown recently that untreated children with CF have a massively increased total bile acid fecal excretion, that is close to 7 times above that seen in a control population; far in excess of the slight increase seen in patients with celiac disease and most other types of malabsorption and only comparable to individuals who have had ileal resection. The mechanisms responsible for the abnormally large bile acid sequestration in CF remains unknown. In preliminary studies of bile salt kinetics by stable isotope dilution techniques bile salt synthesis appears to be markedly elevated in untreated patients with CF and pancreatic deficiency. However, this increase may be insufficient to maintain the bile acid pool size. Administration of pancreatic enzyme supplements is apparently effective in cutting these fecal losses. However, large dosages of enzyme supplements have been reported to produce hyperuricosuria and possibly renal interstitial fibrosis; therefore indiscriminately large amounts of pancreatic enzyme supplements should not be given.

Studies have started to clarify bile acid metabolism in this disorder. Duodenal aspirates for cholesterol and bile acid determinations are being obtained $^{14}C$ - cholic acid and $^{3}H$ - chenodoxycholic acid then are administered and blood, urine, stool, and repeat duodenal aspirates are collected to study the metabolism synthesis, reabsorption and fecal loss of bile salts in these patients. It is also planned to repeat these studies with different dosages of pancreatic extracts in order to determine the optimal amount of pancreatin to be administered in order to avoid side effects and yet correct sufficiently the bile acid fecal loss.

It is thus hoped to clarify not only the bile acid metabolism in CF but also the significantly increased incidence of gallstones in this disorder. Many of the stones in patients with CF are cholesterol, although bilirubin stones have been seen. Cholesterol is solubilized via incorporation into aqueous micelles of bile acid and lecithin. When cholesterol is not dissolved, the bile becomes super-saturated and cholesterol crystals are formed. These crytals accumulate around other bile substances to form gallstones. The decrease in

cholesterol and induce stone formation.

E. Other Clinical Studies

Several other clinical studies have been conducted. A survey of *Pregnancy in CF* has been conducted throughout the country and the results of 129 pregnancies in 100 females with CF are being analyzed. It is felt that pregnancy in CF is hazardous for a mother and the fetus and not advisable unless the lung disease is mild, (NIH Score 80). Antibiotic treatment, often with anti-pseudomonas agents, is absolutely necessary during pregnancy, despite possible hasards, in order to save the mother and the offspring. At the present time no guidelines as to the best way to handle pregnancy in CF are available and these findings will have an important impact on the medical treatment of this disease.

*Syndrome of inappropriate antidiuretic hormone secretion (SIADH) in CF* was described for the first time. It may be more common than is realized in CF and is especially important to recognize promptly in this disease, as the treatment is the exact opposite of that of hyponatremia due to salt loss because of sweating, (fluid restriction, rather than administration). As both circumstances may represent a medical emergency prompt and correct diagnosis may have fateful therapeutic consequences.

With an increasing number of CF patients surviving until adulthood *hypertrophic pulmonary osteoarthropathy* is being recognized with greater frequency in our patients. Three of them have been seen with characteristic X-ray changes but two of them are asymptomatic, although the third has a widespread and disabling disease. Interestingly this patient who is clearly symptomatic had negative X-ray findings when the diagnosis was first suspected clinically. All three patients had severe pulmonary disease and the NIH Clinical Score ranged between 40 and 50.

Various methods of treatment have been investigated. The effects of *Tobramycin*, an analogue of Gentamicin, were assessed and it was not found to be significantly different in the majority of patients from the latter agent. *Ticarcillin*, an analogue of carbenicillin, was also used with success in a number of patients with CF. The advantages of ticarcillin over carbenicillin, although it has the same spectrum as the latter, is that it is more potent and can be given in smaller dosages, thus decreasing the total amount of sodium administered a factor of special importance in patients with chronic pulmonary disease and cor pulmonale. In addition, ticarcillin appears to be less hypokalemic than carbenicillin. *Azlocillin*, a new anti-pseudomonas antibiotic, is now under study. We have shown that vitamin E deficiency which is consistently present in patients with CF and pancreatic deficiency results in a number of subclinical abnormalities and that CF patients should receive supplements of this vitamin. The bioavailability of three different vitamin E preparations were studied. Absorption curves following an orally administered dose of 20 U/kg/body weight were obtained on 7 CF patients and 3 normal volunteers. Plasma tocopherol levels were determined at 0,3,6,9,12,24 and 48 hours. Maximal levels were generally observed approximately nine hours after taking the medication and the 2 water miscible alpha-tocopherol acetate preparations performed equally well indicating that the active form of the vitamin was being absorbed adequately. In contrast the fat soluble form resulted in nearly flat absorption curves in the CF patients and

was clearly less well-absorbed in the normal volunteers. This study is being extended at the present time to include CF patients without pancreatic deficiency. In addition, the possibility of using this vitamin E loading test as a convenient, non-invasive diagnostic test for the evaluation of malabsorptive states other than CF is being studied.

Significance to Biomedical Research: Cystic fibrosis in adults needs further definition, as many of the complications seen in the older age group are different and are treated differently from those in children. In particular, pregnancy in CF is hazardous, and information is being gathered on this point which has posed a serious practical problem for physicians. With the data gathered from a number of CF centers throughout the country the results of a assisted ventilation in CF were assessed they are much poorer than those reported in series of patients with chronic obstructive pulmonary disease of other etiologies. The difference in the type, course, and response to treatment of the diseases may explain this diversity. A number of precipitating factors leading to acute pulmonary decompensation were identified and it is expected that important and useful guidelines as to this type of therapy in cystic fibrosis will be available for the first time.

The studies on altered glucose and bile acid metabolism in cystic fibrosis will have wide implications as the nature of the disorder and as to the practical aspects of treatment.

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(2) di Sant'Agnese,P.A. and Davis, P.B.: Research in cystic fibrosis. N. Engl. J. Med. 295:481-485, 5340541, 597-602, 1976.
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| SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space) | U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT Z01 | PROJECT NUMBER Z01-AM 49003-02-PMR |
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PERIOD COVERED

July 1, 1976 to Sept.30, 1977

TITLE OF PROJECT (80 characters or less)

Cyclic Nucleotides and the autonomic nervous system

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

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☒ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☒ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Cystic Fibrosis (CF) is a disorder primarily involving the exocrine glands, and we have begun to study cyclic nucleotides, which may be essential determinants of secretion in CF. Present topics are: (1) the concentration and distribution of cyclic AMP and cyclic GMP in normal and CF body fluids, (2) the response of cyclic AMP to β receptor stimulation in normal and CF lymphocytes and fibroblasts, the (3) the response of cyclic GMP to stimulatic of the cholinergic receptor in several systems, (4) levels and response to physiologic stimuli of circulating norepinephrine, epinephrine, and dopamine-hydrolxylase, measures of sympathetic nervous system activity in CF, and (5) measurement of number and binding affinity of β-receptors in CF tissues.



PHS-6040
(Rev. 10-76)

Objectives: The role of the cyclic nucleotides in exocrine secretory processes is still being determined, but it is suspected that they mediate the influence of the autonomic nervous system on the glands and may be essential determinants of secretion. Since cystic fibrosis is a disorder involving predominantly the exocrine glands, we have begun to study the physiology and distribution of cyclic nucleotides in CF, and their response in certain tissues to $\alpha$ or $\beta$-adrenergic or cholinergic stimuli.

1. Isoproterenol stimulation of cAMP in leukocytes and fibroblasts.

We have measured the response of intracellular cAMP to stimulation with isoproterenol and Prostaglandin $E_1$ ($PGE_1$) in mixed leukocytes preparations, granulocyte preparations, mononuclear cell preparations, and cultured skin fibroblasts. In all these cell systems, basal cAMP and $PGE_1$-stimulated cAMP are normal in the CF samples, but isoproterenol-stimulated cAMP is greatly diminished in CF cells.

We have shown that this decreased response does not result from kinetic differences or differences in dose-response relations for isoproterenol. Also, it is unrelated to the degree of illness as assessed by NIH clinical score, to the drug regimens of the patients, or the presence or absence of pancreatic insufficiency. From experiments with patients with other pulmonary diseases, it does not seem to be related to lung disease per se.

We have also studied mixed leukocyte preparations from CF heterozygotes and found that isoproterenol-stimulated cAMP is intermediate between normal and CF values. Currently we are in the process of examining the abnormality further by measuring adenyl cyclase activities in broken cell preparations and by measuring the number and binding affinity of $\beta$-receptors. Preliminary measures of cGMP have also been made in this system.

We have also investigated the influence of physiologic concentrations of polyamines on the cAMP-generating system in leukocytes and in our whole-cell preparations they appear to have little effect.

2. Plasma norepinephrine and dopamine - $\beta$ - hydroxylase levels in CF.

Because of the abnormal metabolic response to isoproterenol stimulation, we studied the sympathetic nervous system by an established method. We found that CF patients had significantly lower plasma norepinephrine than age matched controls in both the standing and supine positions. Also the expected increment in plasma dopamine - $\beta$ - hydrolxylase with exercise did not occur in CF patients.

3. Cyclic AMP and cyclic GMP in urine.

Murad et al. have reported abnormalities in cAMP and cGMP excretion in CF, compared to age-matched control ( J. Endocr. & Metab 10:552, 1975). We have determined cAMP and cGMP in 24-hour urine from adult CF patients and appropriate controls and have found that male CF patients have significantly increased urinary cAMP, and cGMP. For females, however, there was such marked variability that conclusions were impossible to draw.

Because of this finding we studied urinary cAMP in three normal women

around the menstrual cycle and correlated this with determinations of urinary estrogens and pregnandiol. We found significant variation of urinary cAMP around the menstrual cycle. We are currently in the process of determining cGMP on these same urine samples and of determining the day to day variation in cAMP and cGMP excreted by males.

No correlation could be determined between the NIH clinical score, a measure of severity of illness in CF, and urinary cAMP or cGMP.

Significance to Biomedical Research: The process of secretion is only now being elucidated and since cystic fibrosis is a disorder predominantly of secretory glands, it is important to study the various parts of this process to define any possible abnormality.

These findings of decreased cAMP response to isoproterenol in both leukocytes and fibroblasts from CF homo and heterozygotes are the first clear suggestions of a subtle defect in the autonomic nervous system or the metabolic responses to it in this disorder. The fact that many obligatory heterozygotes for the CF gene show this abnormal response would indicate it is not secondary to illness. Also, the decreased plasma norepinephrine levels are the first demonstration with modern assay techniques of catecholamine abnormalities in CF.

Publications:

(1). Davis, P.B., di Sant'Agnese, P.A., and Braunstein, M.: Decreased adenosine 3':5' cyclic monophosphate production in response to isoproterenol in cystic fibrosis leukocytes. Fed. Proc.:36:409, (1977).

(2). Davis, P.B., and Chambers, H.S.: Effect of cystic fibrosis and normal serum on $^{3}$H-L-Fucose incorporation in fibroblasts. Ped. Res. 10:364, (1976).

ANNUAL REPORT SUMMARY

CLINICAL HEMATOLOGY BRANCH

## I. Study of Blood Coagulation and Diseases of Hemorrhage and Thrombosis

### 1. A New Method for Purifying and Characterizing Factor VIII and von Willebrand Factor

The material known as anti-hemophilic factor (AHF) contains two biological activities, one is a procoagulant activity called factor VIII (f VIII) which corrects the clotting defect in classical hemophilic plasma and the other activity supports aggregation of platelets by the antibiotic, ristocetin and is known as von Willebrand factor (vWf) because it corrects the deficiency of platelet aggregation in patients with von Willebrand disease. Current methods of purifying AHF are complicated and lengthy, leading to marked loss of f VIII activity and low recovery of vWf. Uncertainties concerning denaturation of the factors during purification hinder clear interpretation of relationships between f VIII and vWf, some suggesting the activities exist as one molecule, others that two molecules are complexed.

We have developed a rapid and simple method for separating high yields of pure AHF from plasma that permits characterizing the nature of the AHF complex for the first time. The method utilizes a controlled pore glass column which permits very rapid separation of large proteins from all contaminating other plasma protein by appropriate selection of a pore size. AHF is a uniquely large protein (MW > $10^6$), hence is excluded whereas all of the smaller plasma proteins diffuse into the glass channels. Because the column is not compressible, material can be pumped through at rapid rate allowing separation within approximately one hour. With this process we obtain pure AHF judged by immuno- and gel-electrophoresis and the yields are at least ten times higher than those previously obtained. By varying pore size and ionic strength, it was found that native AHF at 37° or room temperature is a complex of f VIII-vWf from which low molecular weight f VIII (identified by coagulant activity and specific immunologic reaction) can be separated. The immunologic identification of f VIII was possible by using the unique antibodies that arise clinically in otherwise normal individuals who develop hemophilia. These patients have an antibody directed specifically against f VIII and not vWf. On the other hand rabbits sensitized against AHF, develop antibodies primarily against vWf and not f VIII. Our findings on normal and hemophilic plasma support the concept that f VIII and vWf are distinct components and that hemophiliacs lack the f VIII component by immunologic criteria. Current studies are continuing to further characterize the nature of the two purified molecules that can be separated from AHF.

### 2. Studies of the Biochemical Mechanism Controlling the Platelet Release Phenomenon

In response to stimulation by thrombin, collagen, epinephrine, antibodies, ionophores, and certain nucleotides, platelets release the contents of their storage granules. The substances released, including ADP and serotonin, promote platelet clumping or aggregation which appears to be the first step of

hemostasis following blood vessel injury. The chemical and energetic basis of the release phenomenon in platelets (as well as in other secretory cells) is poorly understood. The current most popular point of view in the case of platelets is that a protein kinase is activated through stimulation of cyclic nucleotide metabolism, and that the kinase activates actomyosin, leading to forceful expulsion of granular content. Aside from marked flux in cyclic nucleotide formation after stimulation and the presence of actomyosin in platelets, the biochemical links in the chain of postulated reactions have not been identified.

During the past year we have studied the platelet release reaction by comparing conditions of release to those required for release of epinephrine from isolated chromaffin granules which have recently been found to involve an osmotic event controlled by permeant anions. The methods of studying the mechanism of platelet release involve use of tritiated-serotonin to label platelet granules, competitive inhibitory reactions between a variety of drugs that block anionic transport (SITS, probenecid, suramin, pyridoxal phosphate, and isethionate) and the agonists that stimulate release, variations in the anionic composition to determine the permeant anion of importance in controlling platelet release, and changes in ionic strength to evaluate the osmotic component of the reaction. The stimulus for platelet secretion was thrombin or the calcium ionophore A23187.

Serotonin secretion from human platelets was found to be inhibited by the anion transport blocking drugs in a competitive manner with respect to A23187 or thrombin in the drug activity sequence: suramin, SITS, pyridoxal phosphate, and probenecid. Tsethionate was inactive. In contrast to chromaffin granule release, platelet secretion was insensitive to chloride and instead was dependent on $OH^-$. Platelet release was suppressed by low pH and inhibition by the transport blocking drugs was competitive only with respect to hydroxyl ions but not chloride. Serotonin release from platelets was also suppressed by increasing extracellular osmotic strength. The relationship between suppression and external osmotic strength was quanitatively similar to that observed in the case of chromaffin granules. We concluded that platelet exocytosis could occur when serotonergic granules were closely juxtaposed to the plasma membrane thus exposing the granule ion transport site to the more alkaline medium. Secretion of serotonin could occur as a consequence of hydroxyl ion transport and osmotic lysis of the granule-plasma membrane complex analogous to the mechanism of chloride dependent epinephrine release from isolated chromaffin granules.

## II. Study of Immunology of Blood Cell Deficiencies

### 1. A Histochemical Test for Bone Marrow Megakaryocyte Maturation and Platelet Production

Although different stages of megakaryocyte (Mk) maturation can be evaluated morphologically after conventional bone marrow staining, the criteria are not precise and pathological conditions may alter the accuracy of interpretation. In order to more precisely define maturation we applied histochemical techniques in attempts to correlate Mk age and function with a chemical reaction that could be used for histologic staining.

Measurement of glycogen or DNA were not rewarding for all stages of Mks contained PAS positive material and had the same degree of polyploidy. Although acid phosphatase (AP) has been shown to be present in the cytoplasm of Mks systematic studies of changes in AP with Mk maturation in human beings had not been done and Mk AP isoenzymes had not been described. During application of cytochemical techniques for AP we found that different stages of Mks identified by conventional morphologic criteria showed differences in their AP activity and that peripheral blood platelets had AP characteristics similar to the cytoplasm of mature Mks.

In determining optimal conditions necessary for the cytochemical demonstration of Mk and platelet AP we found that methanol, a constituent of fixatives commonly used in AP cytochemistry, inhibited Mk acid phosphatase and the degree of inhibition varied with Mk maturity. Immature Mks contained predominantly methanol resistant acid phosphatase and mature Mks predominantly methanol sensitive acid phosphatase. Platelets contain methanol sensitive acid phosphatase similar to mature Mks suggesting that this enzyme was an index of platelet formation by Mks.

Soluble platelet AP showed three bands on polyacrylamide gel electrophoresis visualized by the same reactions applied cytochemically. Two bands accounting for 98% of the platelet acid phosphatase activity were slow moving and methanol sensitive and one fast moving band accounting for 2% of the activity was methanol resistant. Since acid phosphatase that predominates in younger Mks is not sensitive to methanol, methanol resistant platelet acid phosphatase may reflect the amount of that isoenzyme left in Mks when platelets form. The two slow moving platelet acid phosphatase bands probably represent a monomer and dimer of the same isoenzyme.

This information may prove to be a useful index of platelet production. In certain clinical conditions involving rapid platelet turnover there may be discrepancies between morphologic and biochemical criteria of Mk maturity which would be helpful diagnostically. Comparison of Mk, methanol sensitive and resistant acid phosphatase in disorders such as ITP and other thrombocytopenic syndromes may provide clues to differentiating between defects involving platelet destruction and production.

2. Isoimmune Platelet Disorders

In the past year through numerous referrals we have had an opportunity to evaluate further the specific platelet isoantigens that cause neonatal isoimmune pupura and post-transfusion purpura. We continue to find that approximately 50% of suspected cases of isoimmune neonatal purpura are caused by fetal-maternal mismatch of the $PLA_1$ antigen, an antigen which we first described in 1959. Although this antigen is lacking in only 2% of the population, it accounts for at least half of all suspected instances of isoimmune neonatal purpura. This antigen, therefore, appears to be the most antigenic of all platelet antigens, a fact in keeping with observation that first born children are as likely to be affected as subsequent infants. Evaluation of different forms of treatment including exchange transfusion, transfusion of maternal platelets, steroid therapy and massive random donor platelet transfusion suggests that the latter treatment is the simplest and most effective.

Based on review of the numerous cases that we have seen, it appears that a prerequisite for maternal sensitization may be a compatible erythrocyte blood group between mother and child for all mothers that have developed antibodies have had no antibodies directed against fetal red cells. Although platelets have not been found to contain erythrocyte blood groups, it is possible that antigen antibody complexes formed with soluble blood group substances transferred at the same time as platelets may lead to sequestration of fetal platelets and prevent them from becoming sensitizing.

Among the several cases of post-transfusion purpura that we studied this year was a patient we first reported in 1959. The patient required re-operation, and although all platelet groups were matched for transfusion during surgery, a bleeding episode after surgery led to administration of platelets mismatched for the $PLA_1$ antigen. The patient redeveloped severe post-transfusion purpura for a second time. She was effectively treated by use of the IBM blood exchange apparatus which, despite the patient's precarious clinical condition with three replaced heart valves, effected an 80% exchange on three successive days and resulted in immediate clinical improvement and return of the patient's platelets to normal. This observation lends much support to the hypothesis that post-transfusion purpura results from formation of antigen antibody complex between the recipient's antibody and transfused platelet antigen that is absorbed by the recipient's platelets to cause their immunologic destruction.

3. Thrombocytopenia Caused by BCG Therapy

Bacillus Calmette Guerin (BCG) is an atypical variant of the tubercle bacillus widely used for the "immunotherapy" of human malignancy. Although BCG immunotherapy is known to produce a number of symptoms such as malase, fever, rash and adnopothy in recipients, it is usually well tolerated and has not produced life-threatening complications. During the past year it was noted in the surgery branch of NCI that patients given large intralesional doses of BCG for treatment of primary melanoma developed in addition to fever and malaise, a significant platelet depression. Two of the patients who developed wide spread BCG infection developed severe thrombocytopenia (less than $2{,}000/ml^3$) that persisted for two weeks.

We did extensive immunologic and physiologic studies on these patients with the following findings. Bone marrow aspirates and platelet survival studies indicated marked intravascular destruction of platelets in the face of adequate platelet production. Disseminated intravascular coagulation was ruled out by finding normal coagulation tests. Both patients had remarkable similar time sequences with respect to developing thrombocytopenia at the time disseminated BCG infection was first controlled, and both recovered without splenectomy or use of adrenocortical steroids. Extensive tests for circulating antigen-antibody complexes utilizing serotonin release and complement fixation techniques were not successful in detecting specific anti-platelet antibody. However, by comparing the thrombocytopenia of BCG therapy with that of malaria and other infectious disorders it appeared that thrombocytopenia of BCG was a consequence of the infectious disorder subsiding as immunity arose. The most likely explanation appears to be platelet destruction by antigen-antibody complexes formed between immunoglobulins, the constituents of infectious organisms.

| SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space) | U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT** | PROJECT NUMBER ZO1 AM 51,000-20 CHB |
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PERIOD COVERED
July 1, 1976 through September 30, 1977

TITLE OF PROJECT (80 characters or less)

Study of Immunology of Blood Cell Deficiencies

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

| | | | |
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| OTHER: | O.S. Markovic | Visiting Scientist | CHB, NIAMDD |
| | K.M. Tack-Goldman | Investigator | CHB, NIAMDD |

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☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Autoimmunity, drug antibodies, platelet antigens, isoimmunization, transplantation antigens, hepatitis, hemagglutination tests, thrombocytopenia, blood coagulation inhibitors



PHS-6040

Project No. Z01 AM 51,000-20 CHB

Project Description:

Objectives:

To study the nature of immunologic reactions which result in formation of antibodies against autologous blood cells, and to determine the significance of immunity in development of "idiopathic" blood cell deficiency states. Of special interest are the biochemical reactions which result in formation of complexes between cells, antibodies, and drug haptenes and the physiologic processes which result in sequestration of cells with attached antibodies. In recent years our work on identifying inherited leukocyte and platelet isoantigens has led to a study of the significance of these antigens in hetero- and homo-transplantation. Immunologic techniques have been used to develop a number of clinically practical diagnostic tests for certain infectious diseases and drug addiction.

Methods Employed:

Techniques of quantitative immunochemistry, including preparation and physicochemical characterization of purified antibodies and antigens, microanalyses for nitrogen, histamine, and alkaloid drugs, quantitative measurements of complement fixation, cellular agglutination and precipitin reactions, immunoelectrophoresis, methods of provoking antibody responses in man and animals, and isotopic and flourescent labeling applied to antigens and antibodies. Tissue culture techniques, red cell agglutination and techniques and lymphocyte transformation tests, including radioautography. Methods of hemagglutination involving attachment of various antigens to erythrocytes by chemical coupling.

Publications:

1. Norton, J.A., Shulman, N.R., Corash, L., Smith, R.L., Au, F. and Rosenberg, S.A.: Severe thrombocytopenia following intralesional BCG therapy. Cancer, 1977, in press.

2. Markovic, O.S. and Shulman, N.R.: Megakaryocyte maturation indicated by methanol inhibition of an acid phosphatase shared by megakaryocytes and platelets. Blood, 1977, in press.

| SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space) | U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT** | PROJECT NUMBER Z01 AM 51,001-20 CHB |
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PERIOD COVERED
July 1, 1976 through September 30, 1977

TITLE OF PROJECT (80 characters or less)

Study of Blood Coagulation and Diseases of Hemorrhage and Thrombosis

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

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None

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Hemophilia, coagulation mechanism, clotting factor assays, clotting factor purification, platelet physiology, platelet metabolism



PHS-6040

Project Description:

Objectives:

Study of the reactions and interactions of coagulation factors *in vitro* and *in vivo* to define further the nature of the blood coagulation mechanism, to determine factors of significance in the pathogenesis of diseases of hemorrhage and thrombosis, and to develop better forms of therapy for these diseases.

Methods Employed:

Methods of protein purification and characterization, techniques of enzymology applied primarily to proteolytic enzymes and their inhibitors, kinetic analyses of enzyme reactions, procedures for quantitative measurement of various clotting factors, pharmacologic and physiologic techniques applied in man and animals, and assessment of metabolic pathways of blood cells with radioactivity labeled substrates.

Publications:

1. Uhlemann, E.R., TenPas, J.H., Lucky, A.W., Schulman, J.D., Mudd, S.H. and Shulman, N.R.: Platelet survival and morphology in homocystinuria due to cystathionine synthase deficiency. New Engl. J. Med. 295: 1283-1286, 1976.

2. Shulman, N.R.: Factor VIII inhibitors: immunology and therapy. Ann. Int. Med. 86: 606-608, 1977.

3. Pollard, H.B., Tack-Goldman, K.M., Pazoles, C.J., Creutz, C.E. and Shulman, N.R.: Evidence for control of serotonin secretion from human platelets by hydroxyl ion transport and osmotic lysis. Proc. Natl. Acad. Sci. U.S., 1977, in press.

## OFFICE OF THE ASSOCIATE DIRECTOR FOR EXTRAMURAL PROGRAM ACTIVITIES

In FY 1977 two Associate Directors were recruited, one each for the Arthritis, Bone and Skin (ABS) cluster and the Diabetes, Endocrinology and Metabolism (DEM) cluster. This completed the staffing of the Associate Directors for the four program clusters. All Associate Directors have given considerable attention to the realignment of their cluster activities and a structuring of their organizations to meet their programmatic objectives. Recruitment of staff for the expanding programs in the ABS and DEM clusters is being actively pursued.

During FY 1977 the Office of the Associate Director for Extramural Program Activities established and staffed a review unit. Four of the five Executive Secretaries in this unit have been assigned as the responsible review officer for each of the four clusters. This staff will be responsible for the review of program project, core center and National Research Service Award Institution fellowship applications as well as contracts. A major responsibility has and will continue to be the review of large multipurpose disease-oriented center applications. Major focus to date has been on the Diabetes Research and Training Centers, the Multipurpose Arthritis Centers, and the Urolithiasis Specialized Centers of Research. During the spring of FY 1977 this unit reviewed 96 applications in these three programs. To facilitate the review process this Office, through the Institute has requested a project review committee which will act in an advisory capacity to the review unit. Throughout the year staff of the Office of the Associate Director for Extramural Program Activities has assisted program staff in discussions with potential applicants and in the further development, expansion and clarification of the various center grant guidelines.

### Administrative Office

During FY 1977 the travel function continued to dominate the operation of the Administrative Office especially in the use of the Chairman's Grants, this being due primarily to the responsibility for the review function being assigned to the EPA cluster. This resulted in a continual backlog of claims to be processed until late in the third quarter when an additional permanent employee joined the Administrative Office staff. This staff increase (along with the employment of an experienced summer employee) has permitted the Office staff to eliminate the majority of the backlog of travel claims as well as that in other office responsibilities, e.g., procurement, training, files, directives, maintenance, etc. The continuing rapid growth of the staffs of all of the clusters has caused a marked increase in staff requests for services made to the Administrative Office and the critical need for additional space. This additional space must become available in early FY 1978 if work efficiency is to be maintained. The deterioration of the physical plant of the Westwood Building has added to the difficulties experienced in recent months. It is hoped that FY 1978 will see a more concerted effort by the appropriate authorities to upgrade the conditions within the building. Considerable time and effort were devoted to reallocation of space. Reassignment of office space was done in such a way as to have all the offices in a given cluster in a common area.

## Program Evaluation Branch (PEB)

The PEB is responsible for maintaining the Extramural Programs Management Information System (MIS) used by Institute staff as a tool in decision making and for special reporting needs. It is a semi-automated system, using NIH computer facilities.

This year, the conversion to a more fully automated system was begun by PEB staff to increase the use of computers and decrease the need for manual updating, coding and other clerical duties, some of which duplicate the work done in the Division of Research Grants (DRG). This conversion has been hampered because of the press of other priorities, but within the next year we plan to have it operational and eliminate the duplication of effort that was cited in the DHEW audit report of last year.

When completed, more than 60% of the information stored in the MIS of DRG on each National Institute of Arthritis, Metabolism and Digestive Diseases (NIAMDD) grant application will be automatically transferred to the NIAMDD, MIS. We will then add the scientific and other data unique to the Institute's needs.

In the meantime we have made use of other resources including overtime work performed by DRG staff and temporary employment of Dr. Offutt who has been a tremendous asset, working on our large inventory of applications that need to be scientifically coded. We are very grateful for his assistance.

The transitional quarter (July - September 1976) complicated our reporting requirements for the 15 month fiscal year 1976 and the two moves to new quarters during the year did not increase our effectiveness.

However, we did establish a routine computer program to produce mailing labels for staff use. Also, PEB staff has programmed a special report for the Grants Management Branch that can provide them with financial information daily on all paid NIAMDD grant activities. This report is particularly useful near the end of the fiscal year.

We coordinated the Institute's response for the NIH Inventory of Clinical Trials. This year the Institute reported 38 continuing trials and 10 new ones.

Three new technical information specialists were hired in April, one as a replacement and two in new positions.

An internal reorganization of the PEB has taken place this year in response to the Institute's cluster concept. The technical information specialists, under the direction of Mr. John S. Palmer, are being assigned responsibilities over and above the scientific coding of applications. Each specialist is assigned a cluster and is spending time learning that cluster's needs as they pertain to the services the PEB can provide. These duties will expand as time and personnel permit. One aim is to inform the clusters of the facilities available to them and assist staff in their use.

Grants Management Branch (GMB)

During the past year perhaps the most significant development having impact in the area of Grants Management has been the requirement for members of the GMB staff to participate in site visits for the review of program project and center applications sharply increased this year. This was brought about by a combination of factors. The first was the decision for the AM Institute to handle the initial review of such applications, instead of DRG. Further, it was decided that GMB staff would function as the fiscal consultant on such site visits. The other major factor was the center grant announcements for the Diabetes, Arthritis and Kidney programs. Because of the numbers of such applications received, the workload of the branch for site visits and detailed budget preparations was tremendously increased.

One of the complicating aspects evolving from the work of the Diabetes Commission was the concept of "Diabetes Related". The idea of having grants being administered by a program other than the program providing the funds was a new one. GMB, in conjunction with other offices, developed guidelines for the use of Diabetes funds by other programs. We also developed new program classification codes for use on encumbering documents to provide sufficient information to account for "Diabetes Related" funds.

During the year a decision was made to alter the routing of correspondence for the Extramural Programs. Previously, copies of all outgoing correspondence had to be routed through GMB for review. Since GMB staff are preparing more and more letters and countersigning many that are prepared by program staff, it was felt that this review was no longer necessary. Another important criterion in this decision was that better utilization could be made of existing branch personnel. This change will cause a significant drop in the number of official grant files flowing into GMB and will release as much as one-half man year of effort for other purposes.

The EP received a supplemental appropriation amounting to $10.6 million during FY 77. Considerable effort was expended by GMB personnel in the distribution of these funds among the various program areas. We were involved quite closely in consultation with the Director's Office for the distribution and provided the detailed calculations of the various alternative distribution methods.

The personnel situation within GMB, while not substantially improved at the moment, shows promise for the future. This promise is based on permission to recruit one (1) new Grants Management Specialist at the GS-11 level and one (1) clerk typist. However, the recruiting process has proven to be more difficult and time consuming than expected. Ultimately, we plan to assign staff within the branch so that each cluster will have both a senior and junior Grants Management Specialist. While the personnel situation shows these positive signs of improvement, space for the branch now presents a major problem. With the reassignment of space this year, GMB lost substantial usable square footage. With the addition of new personnel, lack of space will become critical.

Operations Office

The extra council this year increased the operations office workload considerably and combined with the move in the spring into smaller quarters, has made the working conditions much too crowded. Therefore, it is planned to change the filing system for the official grant files to mobile units and thereby freeing some floor space for better utilization of the work area.

Associate Director, DEMD

The Associate Director has been planning and directing the programs within the Diabetes, Endocrinology and Metabolic Diseases Cluster with a view toward integrating them into a coordinated effort by placing emphasis on the common aims, goals and opportunities of the individual programs. He has been planning and developing new initiatives, particularly in the area of diabetes, including new research opportunities, the development of manpower and the area of interface between research and delivery of clinical care (e.g., design and implementation of an Education and Information Clearing House).

Diabetes mellitus being the leading medical and public health problem it is, associated with serious chronic complications, concern clearly goes beyond NIAMDD and involves the interests of other institutes. Therefore, the Associate Director has been involved in activities designed to identify shared interests and resources between institutes which can form the basis for cooperative efforts to implement a comprehensive diabetes plan.

The Associate Director is chairman of the Intra-NIH Diabetes Mellitus Coordinating Committee charged with the development of significant intra-NIH relationships for the attack on diabetes mellitus. - He is also involved in the planning and development of a clinical trial for the evaluation of the Relationship of Control of Blood Glucose and the Vascular Manifestations of Diabetes. -

In cooperation with the NHLBI, he has been active in the development of a new support mechanism called "Special Emphasis Research Award: Diabetes Mellitus: Cardiovascular, Metabolic & Endocrinologic Aspects", which has as its goal the creation of a pool of highly qualified investigators with expertise in both fundamental and clinical research disciplines related to the focus of the program.

There have been discussions of the opportunities for cooperating with the Eye Institute in their projected clinical trial relating to diabetic retinopathy.

The Associate Director serves as chairman of the Inter-Agency Diabetes Mellitus Coordinating Committee, is a member of the National Diabetes Advisory Board and sole NIH representative on the Executive Committee of that body. He is also a member of the Research Committee of the American Diabetes Association in which capacity he reviews research grant applications and submissions to scientific journals. In his role of Associate Director, he is frequently and intimately involved with the activities of the Juvenile Diabetes Foundation, the American Diabetes Association as well as with other groups and agencies concerned with diabetes.

A good deal of effort has been directed toward the planning and implementation of an Evaluation of the State of the Art in Endocrine and

Metabolic Diseases within NIAMDD and other institutes. The Associate Director has been working closely with Dr. Sidney Ingbar (chairman of the evaluation effort) with respect to workshops to be held, chairpersons to be selected, choosing the mode in which the information will be collected, organized and presented, and numerous other administrative details involved in such an effort.

Finally, the Associate Director has been active in the recruitment of personnel into the DEMD cluster (particularly the Diabetes Program) to assume the management of all the various new programs and initiatives.

## DIABETES PROGRAM

In FY 77 the Diabetes Program of NIAMDD received from Congress a substantial increase in funds to implement the extensive recommendations of the National Commission on Diabetes. These recommendations called upon the Institute to increase its support of investigator-initiated research into diabetes and related endocrinology, metabolism and nutrition research, to establish a new multipurpose Diabetes Research and Training Centers Program, to expand its training programs and to initiate new programs in diabetes to foster development and maintenance of research resource facilities and the correlation and appropriate dissemination of epidemiological and educational data and information.

The major area of research is in the support of investigator initiated research into the metabolic basis of diabetes and the application of these studies to the causes, prevention and cure of diabetes. Major emphasis is on studies of (1) etiology of diabetes, including the possible role of viruses, immunology and nutrition (2) the structure and function of the pancreatic hormones as well as related peptides and enzymes (3) mode of action of insulin and glucagon, including mechanisms of the derangement of release, secretion and biosynthesis (4) pancreatic islet transplantation (5) artifical devices for the control of blood glucose (6) carbohydrate, lipid and protein metabolism and nutritional interrelationships including obesity and (7) the complications of diabetes.

Early in 1977 the Institute issued a Request for Research Grant Applications in the area of Diabetes and Related Problems. The categories that were identified included the following:

1. Projects directly concerned with diabetes
2. Projects directly concerned with a diabetes - related endocrine or metabolic disorder, and
3. Projects which are not directly concerned with diabetes but which could reasonably be expected to contribute to the diagnosis, treatment, cure, or prevention of diabetes or a diabetes - associated disorder. Over 400 applications were received in response to this request. Those applications will be reviewed by a special Council meeting in August for funding from FY 77 funds.

The Institute also announced the availability of Clinical Investigator Awards which provide an opportunity for clinically - trained individuals with demonstrated aptitude in research to develop into independent investigators. Three of these awards were started as of 7/1/77.

The Commission recognized the close relationship between diabetes and other areas of research particularly endocrinology, metabolism and nutrition, and recommended NIAMDD support of diabetes-related research in those areas. A committee was established at NIH to assist the Diabetes Program in defining "diabetes-related research" which would fall within the purview of other AM Institute programs but could be considered for funding from diabetes funds. The areas that were identified are: research clearly related to diabetes, research that involves regulation of metabolism by hormones such as insulin,

glucagon, somatostatin, growth hormone, somatomedin, catecholamines and gastric inhibitory polypeptides, regulation of glucose or triglyceride metabolism, investigation of the endocrine pancreas, nutrition as it relates to obesity and any research that relates in a major way to some fundamental process having potential bearing on any aspect of the syndrome of diabetes mellitus. About 3 million new dollars and 3.7 million previously identified dollars are now supporting diabetes related grants in other program areas in the AM Institute. These include studies in cyclic nucleotides, lipid and carbohydrate metabolism, ketogenesis, various regulating enzymes, regulation of liver growth and function, hormone regulation of metabolism, obesity, etc.

In the area of etiology it appears that heredity and environmental factors are involved in the etiology and progression of the diabetic syndrome of which hyperglycemia is the unifying phenomenon. There is recent interest in the possibility that abnormal immune response may play a role in the initiation and/or progression of diabetes. A recent report indicated that in streptozotocin-treated diabetic mice there appeared to be a cell-mediated immune reaction directed against the pancreatic beta cell, and the presence of increased numbers of C-type virus in many of the surviving beta cells suggest that a similar series of events may be responsible for induction of juvenile diabetes in the human. The possibility that disturbances in the autonomic control over metabolic homeostasis can contribute to, or be responible for, some aspects of the diabetic state is being investigated. Evidence is evolving that there is a diffenence in the pattern of inheritance between classical juvenile-onset type diabetes and maturity-onset type diabetes. Investigators have shown that pancreatic islet-cell antibodies (ICA) may be detected for up to 10 years before the onset of juvenile-onset diabetes. Patients with maturity-onset diabetes do not appear to have islet cell antibodies.

Although the major hormonal defect in diabetes resides with insulin, it is now clear that diabetes mellitus may be a bihormonal and perhaps trihormonal disease. Work is in progress on various theories about how insulin exerts such diverse but coordinated effects on many different enzymes. One such theory is that the effects of insulin on many rate-limiting enzymes and their metabolic processes can be understood as one underlying molecular mechanism of covalent modification of proteins through the action of phosphoprotein phosphatase. They have established the fact that acetyl CO A carboxylase is phosphorylated when the enzyme is inactivated. Another group studying the hormonal regulation of hepatic gluconeogenesis has results that indicate that pyruvate kinase is an interconvertible enzyme system regulated by cAMP-dependent phosphorylation and that pyruvate kinase is regulated by glucagon and insulin. Recent studies indicate that hormones such as glucagon alter one of the fundamental aspects of cell metabolism, namely energy production through oxidative phosphorylation. In the area of insulin secretion it has been shown that calcium is a critical factor in the linkage of glucose stimulation of insulin secretion. Work is continuing on the role of calcium in this process and also in the relationship of zinc and calcium metabolism in the islet cells. Cyclic AMP has been shown to have a role in the glucose stimulated insulin release from islet cells. Other observations suggest that starvation states have insulin-releasing patterns similar to human diabetes

and that starvation is accompanied by decreased levels of cAMP, therefore, it is suggested that some defect in cAMP response to glucose may be part of the diabetic state. Glucagon excess is characterestic of human diabetes and is considered by some to be of major pathophysiologic importance in the maintenance of hyperglycemia Others consider abnormalities of glucagon to be of secondary importance and one group has shown that hyperglucagonemia characteristic of diabetes is at least in part due to insulin deficiency. They have shown that insulin effect on the exaggerated glucagon response to intravenous arginine differed in the two types of diabetes. Another investigator has produced a high titer antiglucagon serum in rabbits which has been used for RIA and for exploring effects of binding of glucagon in rats. Preliminary results show that this antiserum lowers blood glucose levels in normal rats. Another investigator has used somatostatin to produce a state of insulin and glucagon deficiency and has shown that glucagon plays a vital role in the maintenance of basal glucose production in the dog. The role of insulin appeared to be that of an inhibitor of the stimulatory effect of glucagon on glucose production. The antagonistic action of these two hormones results in fine regulation of hepatic glucose output. These studies were extended to man and it appears that there is a mechanism whereby stimulatory effects of glucagon on hepatic cyclic AMP production are diminished despite continuing hyperglucagonemia.

It is recognized that an alternative technique for the treatment of diabetes is needed since the secondary complications of the disease are not prevented by the use of exogenous insulin. The Institute is supporting research in pancreatic islet cell transplantation. The major problems in this area are the need for immunosuppressive drugs and the problem of harvesting large numbers of viable human cells. Some investigators are attempting to grow the cells in culture and actively to alter the cultured islets prior to transplantation by culturing in the presence of a specific type of anti-lymphocyte serum to reduce the risk of rejection. One group has obtained purified islet tissue by short-term culture of human, dog, and rat pancreata under special conditions and has used cultured pancreatic fragments as an autotransplant in dogs made diabetic. Another grantee has successfully performed an islet autotransplant in a patient who had pancreatitis by digesting the pancreas with collagenase and infusing the dispersed pancreatic tissue into the portal vein of the patient. The patient became normoglycemic in 2 days. This experiment established the feasibility of islet transplantation. The development of an implantable endocrine pancreas was also accomplished by using neonatal rat cells cultured on bundles of artificial capillaries permeable to glucose and insulin but impermeable to larger agents. This was tested in an alloxan diabetic rat and insulin and glucose levels were restored to normal.

Work is also underway on artificial devices such as a sensor for continuous in-vivo monitoring of the blood glucose levels in diabetics. One such sensor has been developed which uses glucose oxidase and catalase entrapped in polyacrylamide gel embedded in a platinium screen used as an anode and a plain screen or a silver - silver chloride reference electrode used as a cathode. This sensor is still being evaluated and it is hoped that it can be incorporated into an implantable insulin delivery system.

A major cause of mortality and morbidity in diabetes is due to destruction

and loss of function of various tissues, the so called diabetic complications. Investigators are looking at the role of sorbitol and the aldose reductase which converts D-glucose to D-sorbitol. They have developed an RIA for human placental aldose reductase and they are testing their hypothesis that aldose reductase serves a signal function or as a glucoreceptor in the beta cell. They have evidence that Alrestatin (an aldose reductase inhibitor) improved painful neuropathy in one patient and improved motor-nerve conduction velocities in two other patients. They anticipate studies of a longer term adminstration of this drug. Recently studies have indicated a striking prevalence of endogenous hypertriglyceridemia (HTG) in the diabetic population as compared to the non-diabetic. This could be an important risk factor for atherosclerotic vascular disease. It has been shown that while diabetes is frequently associated with HTG, genetic HTG does not carry an increased risk of diabetes indicating a segregation of genes for the two. Another recent finding is an abnormally high proportion of Hemoglobin Alc (HbAlc) in diabetics. The abnormal glycosylation during formation of HbAlc is a chemical reaction and might relate to other metabolic and structural tissue dysfunction in diabetes.

Numerous animal models of spontaneous diabetes mellitus have been reported but the Chinese hamster and the sand rat are the only ones with characteristics relevant to the human disease. Recently a Wistar rat model with fatal juvenile-type diabetic syndrome has been identified and correlative morphophysiologic studies are in progress which may help to classify the pathogenesis and etiology of this syndrome. The Institute is in the process of soliciting information about available models in order to determine some means of supporting models necessary for diabetes research. A Sources Sought issuance was recently submitted to the Commerce Business Daily.

## Special Programs in Diabetes

Within the Diabetes Program, centers, clinical trials, and other special programs are grouped under one Program Director. Generally, these are large programs with probable long-term goals and activities.

Prior to the current year, five Diabetes-Endocrinology Research Centers (DERC) have been funded at Vanderbilt University, University of Washington (Seattle), University of Chicago, University of Virginia, and Washington University (St. Louis). During this year another DERC was funded at the University of Pennsylvania. A requirement for the establishment of these centers is a base of high quality on-going biomedical research. Expanded guidelines for "core" center grants (which includes the DERC's) were written and issued during the past year.

A clinical trial, the University Group Diabetes Project, first funded in 1958 was being almost completely phased out during this year. All that will remain is a grant to the Coordinating Center for additional analyses of data and writing for publication. Although best known for its conclusion that patients on oral antidiabetic drugs encounter increased cardiovascular mortality, the continued analyses of the data will yield valuable information relative to the natural history of diabetes.

The Diabetes Research and Education Act (PL 93-354), signed into law in July of 1974, authorized the establishment of Diabetes Research and Training Centers (DRTC), contingent upon the recommendations of the National Commission on Diabetes. The same requirements as in the DERC, for a base of on-going biomedical research of excellence applies. In addition these new centers will include training (of both medical and allied health professionals) and translational (continuing education and model demonstration) components.

For both types of centers, the grant provides for core facilities (shared resources), pilot or feasibility studies (new initiatives), and limited funds for program enrichment, which is intended to strengthen existing programs, increase productivity, and foster generation of new ideas through collaboration among investigators and departments. They are intended to provide an added dimension which includes capability and potential for a net accomplishment greater than that possible from the individually funded research alone. Research projects are not funded by the center grant, and funding must be obtained in the conventional manner. Support of patient care (including patient education) and stipends for trainees are <u>not</u> available through the center grant. In the training and translation components, assessment and evaluation must be included.

Guidelines for the DRTC's were completed in August, 1976. An announcement for DRTC grant applications was issued in September 1976, with applications due January 15, 1977. Fifteen applications were received and are currently being reviewed, and will be presented to a special August Council in order to facilitate funding in FY 77.

Additionally, in response to a recommendation of the National Commission on Diabetes, NIAMDD, in cooperation with the NHLBI, has assembled an ad hoc committee to determine the feasibility and make recommendations regarding the conduct of a clinical trial to evaluate the relationship of control of blood glucose to the vascular manifestations of diabetes. The committee began deliberations in the fall of 1976 and has met 5 times to date. They plan to have completed a draft of their recommendations before the end of this fiscal year.

## Endocrinology Program

The Endocrinology Program supports research in both clinical and basic endocrinology. Studies are supported concerning normal and abnormal function of the pituitary, thyroid, parathyroid, adrenal, pineal and thymus glands. A large share of the program is concerned with the mode of action of hormones, at the cellular and sub-cellular levels, and with the biosynthesis, metabolism and secretion of hormones. Since hormones are produced in glands and are transported in the bloodstream to their target organs, there are numerous studies concerning the binding of hormones to protein carriers in the blood and their subsequent release. Hormone action at the cellular level by binding to cell membranes or to intracellular material is also being studied. Thus, studies of binding mechanisms and kinetics are important within the overall framework of hormonal action.

In order to trace hormonal activity, methods of assay must be developed. In recent years, very sensitive methods such as RIA have been developed. There is a continued effort to improve these methods and to develop new ones. The immunologist and the endocrinologist now work closely together.

It has been known for some years that the central nervous system, primarily through the hypothalamus, affects endocrine function. Many studies are now being supported concerning the structure and function of the so-called "releasing factors", i.e., small peptides produced in the hypothalamus which release or inhibit various pituitary hormones. Interesting recent studies show that some of these peptides are identical to segments of larger hormones. It is thus possible, that a larger pro-hormone molecule can split into fragments having several different hormone actions. Work is progressing to confirm this hypothesis.

The peptides produced by the hypothalamus are generally small molecules (probably fewer than 20 amino acids) and their chemical characterization is, therefore, fairly simple. Some investigators have 'exchanged' one or two of the amino acids making up a peptide and, in some cases, have found that the physiological activity of the hormone has been altered so as to make the effects longer lasting than those of the native hormone. These compounds can in turn be used in studies requiring a prolonged effect of the hormone. Eventually, it is hoped, they may prove useful in the treatment of human disease.

Classically, endocrinology has been concerned with the study of chemical substances produced in certain glandular tissues for transport to target organs where their effect is seen. In recent years, other substances have been shown to have action similar to the traditional endocrine activities, e.g., prostaglandins. The endocrinology program supports studies on prostaglandins as well as on newer substances such as endophins and enkephalins. These substances have been shown to have hormone-like activity but appear to be produced in the brain. They appear to have a more restricted area of activity, that is, their effects often are manifested in the general area of production rather than at distant sites. Interesting new work concerning these substances is expected in the future.

The following are specific examples of research supported by the program, a list which is by no means exhaustive.

Thyroid hormone metabolism during liver regeneration: Following partial hepatectomy, serum $T_4$ and $T_3$ levels fall in proportion to the amount of liver tissue excised. Other stressful procedures do not cause this decrease. It appears that at least 3 phenomena are involved: 1) increased utilization and turnover of thyroid hormone by the regenerating liver; 2) diminished hormone secretion by the thyroid gland between 6 - 12 hours after surgery, and 3) a slight reduction of serum iodothyronine carrier proteins. These results support the concept that liver participates in metabolic regulation of $T_3$ and $T_4$ and that these hormones in turn help control hepatocellular growth. The researchers suggest that other factors, as yet unknown, control increased hepatic-thyroid hormone turnover following partial hepatectomy.

Studies on somatostatin: Somatostatin is a peptide inhibitor of growth hormone, of the secretion of insulin and glucagon and various gastrointestinal hormones. Analogs to somatostatin are being prepared which, it is hoped, will make it more potent or longer lasting, or will make the hormone selective in its activity. In addition, studies are being carried out showing that somatostatin produced in the pancreas has an effect on insulin and glucagon production. In the Endocrinology Program, studies of this type are an example of 'diabetes-related' research, demonstrating the importance of sound basic studies in several disciplines to the control and eventual cure of disease.

A series of studies has been done on the mechanism of action of steroid hormones at the subcellular level. One of the effects is an increase in the synthesis of new messenger RNA, which appears to be a primary event, initiating most, if not all, of the biological effects of steroid hormones. This is considered important in the understanding of the action of steroid hormones, especially glucocorticoids which are useful in suppression of cancer, of immune response (to organ transplants) and in the suppression of abnormal inflammatory responses such as are found in rheumatoid arthritis.

This Institute has supported the National Pituitary Agency for many years. The agency was originally started to collect pituitaries from human cadavers for the extraction of growth hormone. This hormone was used in studies on growth-retarded children who appeared to lack the hormone. Over the years, the agency has evolved and is now producing and distributing other human pituitary hormones as well as animal pituitary hormones. The agency has provided a work- and time-saving service to the scientific community. The program is being supported by contracts exclusively, thus allowing greater control of the production and distribution of the products. In addition to hormones, their antisera are distributed to qualified investigators for use in RIAs. This program is considered to be very important to the research goals of the Endocrinology Program enabling, as it does, the use of standardized products by the scientific community.

## MANPOWER DEVELOPMENT PROGRAM

Within the Diabetes, Endocrinology and Metabolic Diseases Cluster, training activities are grouped under one program director. These include National Research Service Awards (both Individual Postdoctoral Fellowships and Institutional Training Grants), and Research Career Development Awards. The newly established Clinical Investigator Awards remain within the individual programs.

Under authority of Section 472 of the Public Health Service Act as amended (42 USC 4821-1), the National Institutes of Health (NIH) provides National Research Service Awards to postdoctoral individuals for training experiences in specified areas of biomedical and behavioral research, as well as grants to eligible institutions to develop or enhance research training opportunities for individuals selected by them who are interested in careers in specified areas of biomedical and behavioral research.

For fellows, the proposed study must encompass biomedical research training with an opportunity to carry out supervised research in the specified areas, and offer opportunity to research health scientists, research clinicians, etc., to broaden their scientific background, or to extend their potential for research in health-related areas.

In the grants to support training programs in institutions, pre- and postdoctoral trainees may be supported if either or both level(s) of training are justified in and approved on the basis of the application. The applicant institution must have, or be able to develop the staff and facilities required for the proposed programs.

The National Institutes of Health offers Research Career Development Awards to foster the development of scientists with outstanding research potential who require additional training and experience in productive scientific environments in preparation for careers of independent research in the sciences related to health. Awardees are expected to devote their entire professional effort to the achievement of the objectives of the Research Career Development Award. Their principal involvement must be with the actual conduct of research.

In November 1976, the National Institute of Arthritis, Metabolism, and Digestive Diseases, announced the availability of NIAMDD Clinical Investigator Awards. It enables candidates to investigate, for up to three years, a well-defined problem with a sponsor (or sponsors) competent to provide guidance in the chosen problem. It is anticipated that this award will provide research support in the transition between fellowship or trainee experience and a career in independent investigation. The Clinical Investigator Award differs from the NIH Research Career Development Award in that it specifically seeks to develop research ability in individuals with clinical background and training.

At the present time the DEM cluster is supporting 83 National Research Service Awards for Individual Postdoctoral Fellows of which 28 are in the Diabetes program, 21 are in the Endocrinology program, and 34 are in the Metabolic Diseases program. In addition, a few (10) now phased out Postdoctoral Individual Research Fellowship Awards are still also supported by the cluster.

A total of 32 Institutional Training Grants are being supported by the cluster, of which nine are in the Diabetes program, 16 are in the Endocrinology program, and seven are in the Metabolic Diseases program. Some of the former type of training grants are still active, however, there was no funding for these grants this past year.

The DEM cluster supports 56 Research Career Development Awards of which 18 are in the Diabetes program, 19 are in the Endocrinology program, and 19 are in the Metabolic Diseases program. Some Research Career Awards, which are being phased out, are still supported; there is one in the Diabetes program, eight in the Endocrinology program, and five in the Metabolic Diseases program.

Since the CIA program has been so recently initiated, there are no current awards. However, grant applications are under review at the present time.

It is felt that the creation, within the DEM cluster, of a special program director for Manpower Development will aid greatly in the coordination of efforts in this area. Since the programs of the DEM cluster are so closely related, this arrangement has the particular advantage in allowing for cross-coordination among the programs in the area of manpower and development.

One result of this arrangement, has been to focus more clearly the need for a young investigator award. Initiation of such an award is being explored.

## Metabolic Diseases Program

The metabolic diseases program supports research directed toward understanding cystic fibrosis, Lesch-Nyhan Syndrome, gout, glycogen storage diseases, homocystinuria, and Wilson's disease. In addition, the program supports basic research that although not specifically directed to the understanding of any one particular disease process, serves to foster knowledge development that impacts upon some of the most prevalent and serious public health problems of the day, e.g., diabetes mellitus, atherosclerosis, cancer, obesity.

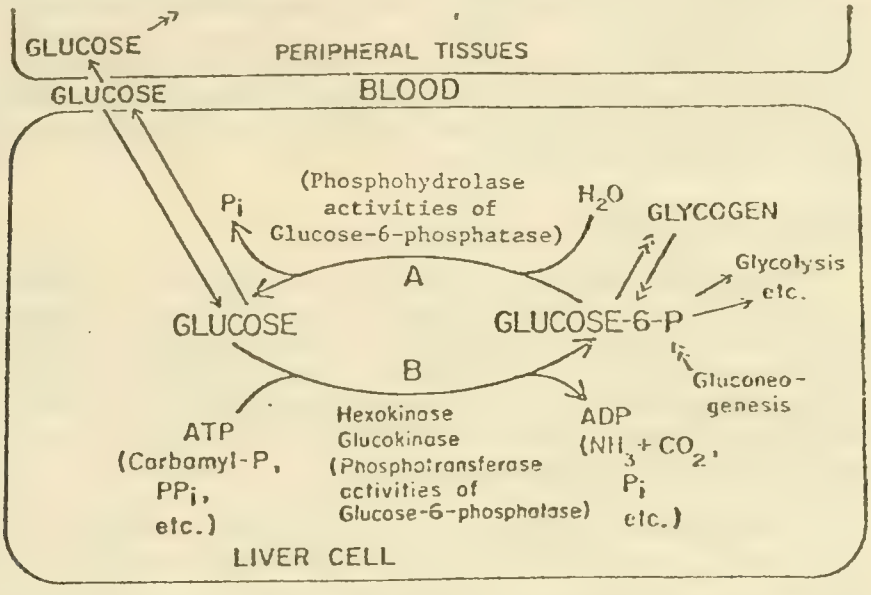
The satisfactory solution of the categorical health problems of the NIH is dependent upon the development of a critical mass of knowledge concerning normal life processes at the molecular, cellular, and organismic levels of organization. To date, research achievements, largely as a result of NIH support of the basic sciences, have expanded our knowledge of structural and functional relationships between molecules, cells, and tissues. Knowledge development is now reaching a critical point where investigators are beginning to examine and unravel the cellular regulatory mechanisms which control and coordinate the diverse cellular functions into an integrated, well functioning, homeostatic organism. Evidence demonstrates that most pathological states are the result of abnormal function of the various bioregulatory mechanisms, e.g., cancer is the result of uncontrolled cellular growth rate, cystic fibrosis is characterized by abnormal control of exocrine gland function. Recognizing the importance of control processes, the metabolic diseases program continues to support projects directed toward increasing our knowledge of cellular control mechanisms. A summary of the significant achievements of the program during fiscal year 1977 is as follows:

### Carbohydrate Metabolism:

While only accounting for one percent of the total body weight, the chemical breakdown (catabolism) of carbohydrates by cellular processes supplies the majority of chemical energy used by the cells. Abnormalities in the control of glucose metabolism lead to severe pathological alterations, including diabetes and hypoglycemia. The body's reaction to exercise and stresses such as excitement, danger, and fright is an outpouring of glucose from the liver. This glucose provides fuel for the body to respond properly to exercise and stress. One of the major factors involved in this glucose response is a special division of the nervous system called the sympathetic nervous system. The sympathetic nervous system affects the liver and other tissues because it releases epinephrine and norepinephrine from its nerve endings and from a special gland called the adrenal medulla located above each kidney.

For many years, scientists thought they knew how epinephrine and norepinephrine caused the liver to release glucose, but recent discoveries by NIAMDD supported investigators have shown that the original scheme is not the major way by which these agents work. Such research suggests that epinephrine and norepinephrine increase the output of glucose from the liver because they raise the level of unbound calcium in the liver cells and this increases the activity of certain catalysts (enzymes) involved in the conversion of stored glucose (glycogen) to free glucose. This new scheme of epinephrine and norepinephrine action may apply to certain other parts of the body on which the sympathetic nervous system acts.

Other Institute supported investigators have established new and expanded roles for the classical enzyme "glucose-6-phosphatase." Institute supported investigations reveal both an expanded distribution and additional catalytic functions for this enzyme.




A controlled balance between glucose-6-phosphate breakdown and glucose-6-phosphate formation in liver is believed to play a prime (but not exclusive) role in the maintenance of blood sugar concentrations at near constant, though different, values in various states of health, nutritional deficiencies, and other diseases. Hepatic glucose-6-phosphatase catalyzes the former reaction, while the enzymes hexokinase and glucokinase have for the past 20 odd years been considered enzymes of glucose-6-phosphate synthesis. Institute supported investigators indicate that synthetic (phosphotransferase) activities of glucose-6-phosphatase (i.e., carbamyl phosphate: glucose phosphotransferase and inorganic pyrophosphate: glucose phosphotransferase) may play a role in glucose-6-phosphate synthesis in liver, functioning in conjunction with hexokinase and glucokinase. Current studies in which glucose utilization and production have been measured in perfused livers and isolated rat liver parenchymal cells, strongly support this concept.

Glucokinase requires insulin, and is thus depressed or disappears in diabetes mellitus or after fasting; synthetic activity of glucose-6-phosphatase increases two to three-fold in such cases. On the basis of

these observations and fundamental differences in catalytic characteristics of these two enzymes (the concentration of glucose required to "saturate" carbamyl phosphate:glucose phosphotransferase is about three to four times that for glucokinase), these investigators have proposed that the ratio, carbamyl phosphate:glucose phosphotransferase/glucokinase, may be a fundamental factor in the establishment of normal blood glucose levels, and in the "retuning" or readjustment of these levels as the physiological state of the individual requires. For example, in untreated diabetes (where unusually high levels of blood sugar are essential for continued life), experimental evidence indicates an increase in this ratio. Insulin administration lowers same, and concomitantly reduces blood glucose levels. Other evidence suggests that alterations in this ratio may be especially important in idiopathic hypoglycemia.

These studies raise the distinct possibility for the therapeutic readjustment of blood sugar levels in the clinical setting by the administration of certain drugs which may alter functional levels of synthetic and hydrolytic activities of glucose-6-phosphatase and/or glucokinase. In this regard, certain of experimental findings recently have been utilized, with apparent success, as the basis for the treatment of a young patient with aberant liver glucose-6-phosphatase and accompanying chronic low blood sugar levels.

Cyclic Nucleotides:

During the process of evolution, cells have evolved common intracellular messengers for precisely regulating the response of that cell or tissue to a specific stimulus. These intracellular messengers include the two cyclic nucleotides, cyclic AMP and cyclic GMP, as well as certain cations such as calcium and magnesium. Dr. Earl Sutherland received a Nobel Prize for establishing that cyclic AMP was a universal messenger for hormone action.

Since Dr. Sutherland's pioneering work, research in the area of intracellular control mechanisms has accelerated at a logarithmic pace because it has become obvious that the cyclic nucleotides are involved in basically all cellular mechanisms, i.e., cell growth, cell movement, hormone secretion, cellular transport, antigen antibody recognition, just to name a few.

One group of institute supported investigators has approached the problem of the role of cyclic nucleotides in normal and abnormal cells by utilizing antibodies that were generated to the cyclic nucleotides. These antibodies are utilized both for total tissue measurement and for locating histochemically where the nucleotides are bound in the cells, i.e., to which specific cellular structures. These experiments are conducted after hormones which are known to produce a given affect on that tissue are added. It is believed that this work can be ultimately applied at the ultrastructural level.

Despite at least 10 years of research by many groups, there is no clear evidence that cylic GMP is by itself a messenger for hormone action. However, these investigators have found by immunocytochemical

techniques many examples of changes in cyclic GMP localization. After hormonal stimulation, for example, cyclic GMP nucleotide is involved in the action of myosin, a contractile protein present in all cells, in hormone secretion (insulin, glucagon, etc.), and in calcium movement across membranes. These same investigators are currently focusing on human red blood cells (which contain a myosin-like protein called spectrin), because it is believed that changes in cyclic nucleotide metabolism are important in these cells for such functions as oxygenation, transport, and the control of cell shape. When they have elucidated mechanisms of control in normal cells, they will then focus on diseases such as sickle cell anemia and hypertension.

Additionally, this histochemical work has shown that cyclic GMP is involved in nuclear regulation in all cells. They are attempting to elucidate its function in the nucleus. They believe such studies will help in unraveling the abnormalties that occur in cancerous cells.

Another group of NIAMDD supported investigators is attempting to determine what factor controls cyclic AMP metabolism in intact cells. They are using as models the highly characterized cultured human lung fibroblasts, WI-38 and its aberrant virus transformed counterpart, VA13.

Thus far, these investigators have found that there are a variety of defects in the ability of the transformed cells to regulate cyclic AMP levels properly. For example, although both cells demonstrate increased cyclic AMP levels in response to prostaglandins and catecholamines, the transformed cells respond more dramatically, demonstrating a greatly prolonged increase in cyclic AMP levels and less specificity towards agonists (cyclic nucleotide phosphodiesterase). The transformed cells contain less of the enzyme system which destroys cyclic AMP and may be unable to regulate this system. In addition, the VA13 cells are less able to dampen the action of agonists by extruding cyclic AMP into the medium. Conversely, no unique differences in either the cyclic AMP activated or independent protein phosphokinase activities of the two lines are obvious.

These same investigators have also studied the alledged influence of modified cyclic AMP levels in the aberrant growth of the transformed cells. To date, using a wide variety of approaches, they have been unable to obtain evidence to support the notion that there is a direct relationship between basal cyclic AMP levels and growth control. They have reached the tentative conclusion that cyclic AMP is involved as a fine control over differentiated functions, and is probably not involved in the control of cell growth in any direct way.

The health related aspects of research in cyclic AMP metabolism are many and varied, for it is a very fundamental regulator of cellular activities. The most obvious situations are a variety of endocrinopathies, including diabetes mellitus. However, it seems likely that cyclic AMP may be involved in such diverse problems as diseases of the cardiovascular system on the one hand and cholera on the other. Only as the nature of the second messenger systems are better understood can the derangements underlying the pathology be understood and rational therapy be developed.

Glycoproteins:

Glycoproteins form an important group of macromolecules with diverse biological functions such as blood group substances, blood proteins, antibodies, membrane proteins, mucins and other tissue secretions and hormones. These are also associated with several pathologic states. Alteration in cell surface glycoproteins and glycolipids during malignancy is a well recognized fact. The enzymes, glycosyl transferases and glycosidases are responsible in living systems for the synthesis and degradation of glycoproteins respectively. These enzymes, therefore, can be exploited with in vitro studies of the normal and diseased state glycoproteins. Institute supported investigators have developed simple procedures for the isolation of several glycosidases from a bread mold (aspergillus niger), pinto beans, deep sea clams and worms. These enzymes have been applied successfully to the structural determination of human chorionic gonadotropin (hCG), a hormone isolated from pregnancy urine. Experiments are underway to extend these studies to hCG from cancerous patients such as those suffering from choriocarcinoma and hydatidiform mole in which the hormone is produced in abnormally large quantities. Furthermore, these investigators have isolated mucins from submaxillary secretions of normal and persons with cystic fibrosis. In order to determine whether the primary disorder in this disease resides in the carbohydrate part, which forms 80 percent of the molecule, the carbohydrate structures in the normal and abnormal mucins with the help of these enzymes are under investigation.

Another group of investigators has directed efforts mainly towards the mucous glycoproteins lining the gastrointestinal and pulmonary tracts. Sugar chains are removed from the protein portion and in many cases the chemical structures and functions are now known. For example, sugar structures on red blood cells determine the blood type A, B, or O. This NIAMDD supported laboratory was the first to demonstrate the biosynthesis of the blood type A oligosacchride.

A real understanding of the significance of glycoproteins in health and disease will be achieved by careful studies into the chemistry and biosynthetic mechanisms regulating the types and amounts of these macromolecular components.

Protein Synthesis and Secretion:

Approximately 50 percent of all the organic material within the human body consists of proteins. The proteins play a role in many of the structural and functional activities of the body.

The plasma proteins participate in the nutritional, coagulation, acid-base regulation, and immunological responses of the organism. One class of plasma proteins, the albumins, are produced within the hepatic cells and are subsequently released into the blood. Because of the functional importance of these plasma proteins, the metabolic diseases program supports programs directed toward increasing the understanding of the mechanisms by which these proteins are produced with an organ and subsequently secreted into the blood.

One of the most accessible systems for studying protein secretion is that concerned with serum albumin synthesis in liver. This system amounts to about one fifth of the total protein produced by liver. With funds granted from the NIAMDD, investigators have recently established that a hitherto unsuspected precursor to serum albumin is found within the vesicular secretory apparatus of rat liver. They have established that its structure consists of a highly basic hexapeptide (a linear sequence of six amino acids) attached to the amino terminus of the serum protein (a single polypeptide chain of 575 residues). Proalbumin is only found within the cell; cleavage of the "pro" appendage coincides with secretion from the cell. These investigators now find this type of albumin present in the livers of other species: bovine proalbumin has the same six residues at its amino terminus. It is expected that the same phenomenon will be found in human liver and that the protein will have the same "pro" appendage as the rat and bovine proteins.

It is of interest to note that an unrelated (secreted) protein, parathyroid hormone, has a precursor with a homologous hexapeptide at its amino terminus. Therefore, this is probably a general phenomemon. Understanding the function of proalbumin may lead to an unravelling of novel aspects of the general machinery involved in secretion (and perhaps control of biosynthesis) of not only albumin but a great variety of the secreted proteins involved in human biology.

Cell Structure:

The boundary of every living cell is the plasma membrane. For decades, this membrane was thought to be an essentially inert physical barrier which played a relatively insignificant role in cell metabolism and function. This view has undergone a complete change during the past 10 years, and the plasma membrane is now known to be intimately involved in regulating cytoplasmic and nuclear functions. This membrane permits the entry and exit of some substances, such as nutrients and waste products, but not a multitude of others involved in the intermediary metabolism of the cell. It controls the shape, form, and movement of the cell. It receives innumerable signals from the environment and selectively transmits the appropriate information to the cytoplasm and nucleus. Receptors for hormones, such as insulin and glucagon and many others, are located in this membrane. Plasma membranes of nerve and muscle conduct electrical signals essential to the functions of such cells. The molecules by which cells recognize their environment and each other are present in the plasma membrane All of these functions of the plasma membrane and many more can be shown to be directly involved in normal and abnormal cell functions, i.e., health and disease. Major changes in structure and/or function of this membrane have been shown or are thought to occur in diabetes, obesity, cancer cells, cystic fibrosis, neuromuscular diseases, and abnormally developing embryos. Research designed to deal with health problems in these and others areas is hampered by the fact that knowledge of membranes is meager at the present time—membrane biochemistry is in fact one of the frontier areas in biomedical science.

Because proteins are the substance that quite generally carry out the specific and sensitive functions of the membrane and proteins, one NIAMDD supported program of research has focussed on membrane proteins. By their very nature, membrane proteins are poorly soluble in aqueous media and thus

not amenable to the techniques developed in the past for the study of water-soluble proteins. Detergents have proved to be the key to success in overcoming this problem. Detergents are structurally similar to membrane phospholipids and make it possible to produce water-soluble protein-detergent complexes in which the protein has retained its native characteristics. These investigators recently have succeeded in preparing the calcium pump protein from sarcoplasmic reticulum (an important control factor in muscle action) in water-soluble and still active and stable form, something that had not successfully been accomplished. This makes it possible to characterize this protein and to study its role in muscle control by standard and proven methods that could not be used without solubilization in detergents.

Other discrete cellular constituents are believed to carry out discrete biological functions in the cell. One group of NIAMDD supported investigators are attempting to understand the role of the small cellular constituents, the lysosomes.

The investigators have uncovered a mechanism in liver in which cells break down their own protein and other cytoplasmic constituents in response to the need for amino acids in the body. This process is carried out within lysosomes, which are small digestive organelles that have long been known to serve as intracellular scavengers. This function differs, however, in that it is a continuous process and is physiologically regulated by plasma amino acids, insulin and glucagon.

As presently envisioned by these investigators, the cytoplasmic mass of hepatocytes, which increases rapidly in response to feeding, subsequently loses protein-containing constituents to maintain a normal circulating level of amino acids. Evidence has been obtained from electron microscopy and from experiments with radioactively-labeled protein that proteins bound to or in the vicinity of smooth endoplasmic reticulum are mainly involved when the demand for amino acids is small, but that most other cytoplasmic components are susceptible to lysosomal attack. Extensive degradation might occur, for example, in severe diabetes where, in addition to a strong need for amino acids, the normal restraint by insulin on this process is lost.

While nothing is known of the way in which this degradative mechanism is regulated by amino acids and hormones, it is clear that portions of the cytoplasm must first be isolated from the rest of the cell by membranes which probably arise from Golgi vesicles. The vacuole thus formed subsequently acquires digestive enzymes by fusing with lysosomes. Degradation then proceeds much like gastrointestinal digestion. These investigators have estimated that this process could account for a major fraction of normal protein turnover in liver and would serve as an important defense against deprivation by maintaining an adequate suppy of amino acids for such essential body services as protein synthesis and the production of blood glucose in periods between food intake.

Diabetes Related Research:

The metabolic diseases program supports numerous projects directed toward understanding the etiology and pathogenesis of diabetes mellitus.

One such group of studies involves the use of adipocyte for a model to investigate the mechanism of insulin action by biochemical and morphological approaches. Studies on the ultrasturctural distribution and behavior of the insulin receptor using a ferritin-insulin complex showed the insulin receptor to be uniquely arranged in naturally occurring small groups on the surface of the cell and differ greatly in behavior from other surface receptors. This provides a structural model for the biochemical data concerning insulin receptor interactions. Biochemical studies have revealed that insulin directly alters an ATP utilizing enzyme on the plasma membrane. The transfer of the terminal phosphate to a yet unidentified membrane component is an insulin sensitive step. Additional studies have shown that insulin treatment of the adipocyte causes profound and unique shifts in the subcellular distribution of calcium. These data suggest that calcium plays a major role in the mechanism of insulin action. The following scheme has thus been suggested for the mechanism of insulin action: insulin interacts with its membrane receptor resulting in activation of a membrane enzyme system that produces a second messenger. This compound alters subcellular distribution of calcium which regulates various kinases and phosphatases that control the enzymes in various metabolic pathways that are ultimately sensitive to insulin and produce the hormones' cellular metabolic effects. These studies contribute to the basic knowledge of cell organization, structure and function which are essential to the understanding of the abnormalities of diabetes mellitus and obesity where alterations in the actions of insulin are implicated. Determining the site of these defects may enable the development of therapy to bypass the defects and hopefully prevent the damaging complications of these diseases.

Metabolic Diseases:

In addition to the support of basic programs, the metabolic diseases program supports research directed toward understanding cystic fibrosis, Lesch-Nyhan Syndrome, gout, glycogen storage disease, and Wilson's disease.

Cystic fibrosis is the most common lethal genetic disease among Caucasians. Estimates are that one infant in every 2,000 live births is affected. There are approximately 30,000 children or young adults afflicted with cystic fibrosis. Estimates indicate that there are 10,000,000 carriers of the disease.

The NIAMDD supports numerous programs attempting to elucidate the metabolic basis of cystic fibrosis. At present, the basis of cystic fibrosis remains unknown. Speculation about the presence of defective protein, glycoprotein and macroglobulins exist while abnormalities of specific enzymatic activities have been detected in a variety of experimental situations. Problems related to the synthesis of proteins and glycoproteins, the transport of these substances to the cell membrane, or their eventual release through the cell membrane are receiving careful scrutiny by NIAMDD supported investigators. The relationship of these molecular abnormalities to polyamines, the salt and water transport, and to intermediary metabolism are under investigation.

In 1977, the Cystic Fibrosis Foundation was awarded a contract by the NIAMDD. The purpose of this contract is to prepare a report defining

accurately the current state of art relative to a broad range of CF-related research activities. It is believed that the contract participants will develop data helpful for defining opportunities for new initiatives and for projections of future NIAMDD activities. The Institute believes that such a document will be useful in its planning activities.

With respect to the ongoing research, numerous investigators have reported the presence of cationic "factors" that are believed to be responsible for the alteration in electrolyte transport associated with cystic fibrosis as well as the dyskinesia associated with mucociliary action in cystic fibrosis patients. NIAMDD supported investigators have postulated that the levels of these proteins are elevated in cystic fibrosis patients as a result of a deficiency of a proteolytic enzyme which hydrolyzes and inactivates the component in normal tissues. These investigators have now demonstrated that arginine esterase, a proteolytic enzyme, is deficient in the plasma and saliva of patients of cystic fibrosis. It has been shown that the reduced enzymatic activity of the arginine esterase is associated with an alpha-2 macroglobin complex. It is postulated that the deficiency of arginine esterase activity is related to the elevation of the levels of polypeptide or protein factors. This is the first report in which there is demonstration of a specific enzyme deficiency associated with cystic fibrosis. The ability to characterize the documented deficiency of arginine esterase and to determine the relationship between the isoenzymes in normal individuals as well as patients with cystic fibrosis should be useful in developing accurate and simple methods to detect patients with the disease, permitting identification of heterozygotes, providing a specific marker which might explain the clinical manifestations of the disease, providing the basis for rational definitive therapy for the disease, and developing accurate approaches for prenatal detection of this disease.

Investigations such as these may give some insight as to the nature or the cause of the basic defect associated with cystic fibrosis.

Cystic fibrosis is considered to be a generalized disorder of exocrine gland function. Fundamental to understanding cystic fibrosis, is the need to develop new information concerning the secretory physiology of exocrine glands. One group of NIAMDD supported investigators have been able to develop reliable and reproducible methods for the isolation of secretory acinary cells from the parotid gland of rats and man, utilizing enzymatic and mechanical forces to disperse and isolate the secretory acinary cells. The cells appear to be morphologically and functionally intact, and their membrane receptors for the neurotransmitters controlling parotid function appear likewise to remain intact and sensitive to stimulation. These same investigations have been carried to human parotid acinar cells obtained from patients with cystic fibrosis and from cadaver subjects without cystic fibrosis, and some of the physiological and biochemical abnormalities have been characterized in these cells. An additional significant methological accomplishment of this laboratory has been the development of long-term cultures of dispersed parotid acinar cells. This system will provide the capability of storing cells for future use, particularly in the case of cells from the human parotid which become available only sperodically.

The use of such methods will improve markedly our knowledge of the cellular physiology of secretion by providing direct information obtained from the studies of isolated rat parotid acinar cells immediately after dispersion or after preservation by cell culture. The direct study of cells from human parotids will provide additional information about the cell physiology of secretion of human parotid in the presence or absence of cystic fibrosis. The cultured cells will provide not only an opportunity to study processes that take a significant length of time but also an opportunity to induce changes resembling those of cystic fibrosis in the cultured cells. It is hoped that these studies will provide important information necessary for the understanding of the pathophysiology of cystic fibrosis and other disease states affecting exocrine glands.

In addition to the evaluation contract and research support program, the NIAMDD is co-sponsoring with the Cystic Fibrosis Foundation, a workshop entitled, "Tissue Culture and Cystic Fibrosis." The Institute believes that such activities will serve to bring new researchers into the area of cystic fibrosis research.

## ARTHRITIS PROGRAM AREA

The research grant and career programs for fiscal 1977 were supported at a level of $14.7 million and the total commitment for training was $0.986 million. Comparable support for the first twelve-month period of 1976 was $13.9 million to support 195 research and career development programs, representing only a 5% increase for these areas. Total training funds available for FY 1976 were $0.473 million to support 14 institutional training programs and 16 individual fellowships both under the National Research Service Act. The complexities of funding for training during FY 1976, which carried into FY 1977, have created an apparent 52% increase in training support. However, $165,000 was the total available for both institutional and individual fellowship awards during FY 1977, which permitted award of only three new individual fellowships and four new training programs in arthritis with less than the desired number of training positions in the institutional program.

Still to be committed is $2.5 million for Multipurpose Arthritis Centers and $0.3 million is being reserved to support the third year of a research demonstration and dissemination project concerned with a computerized national clinic disease databank system for arthritis. Funds have been set aside to support a new clinical investigator award program limited to promising clinically trained individuals with demonstrated aptitude in research, to develop into independent biomedical investigators in the field of arthritis. It is hoped that four or five superior candidates can be identified for this award.

Budget allocations within the arthritis program are being made in five areas which consume 70% of the total research funds available to support programs in: Muscle and Neuromuscular Research (20%); Collagen Studies (16%); Research in Immunology associated with Rheumatic Diseases (15%); Connective Tissue (11%); and Systemic Lupus Erythematosus (8%). Approximately 6% is allocated for research programs in Rheumatoid Arthritis and Degenerative Joint Disease.

A descriptive survey of the major research programs associated with the Rheumatic Diseases follows:

Rheumatoid Arthritis: The general problem of the pathogenesis of inflammation in rheumatoid arthritis is being approached through the elucidation of mechanisms regulating the release of lysosomal enzymes and the determination of their effects on proteoglycan derived from articular cartilage. The structure, function, and biological properties of immunoglobulin peptides continue to be of interest along with the characterization of factors elaborated by activated lymphocytes and studies of delayed hypersensitivity in patients with rheumatoid arthritis. A connective tissue-activating peptide extracted from cultured fibroblasts derived from rheumatoid synovial membranes is being purified to permit extensive characterization and study of the activating process. Other activities are concerned with attempts to demonstrate specific antigens in cultured synovial fibroblasts, the high levels of rheumatoid factor, and immune complexes in patients with rheumatoid arthritis with the intent to characterize these substances and examine biological relationships.

Degenerative Joint Disease: An evaluation of a disease model in the canine shoulder using the German Shepherd with its high spontaneous incidence of an osteoarthritic lesion in the humerus is being conducted. It will use motion pictures, force plate data, contour data and recorded data. Results will be summarized by computer reconstruction of experiments. Amputation specimens, histological studies and a model system will be used to study the correlation between the contact area of the human ankle joint, the load borne by it and the pattern of degenerative arthritis occurring. Characterizable synthetic surfaces in their natural state and after modification are being employed to measure the lubricating ability of synovial fluid in simulating natural and disease states. Finite element stress analysis is being used to evaluate normal and osteoarthritic human knee joints as well as those with total prosthesis. Normal and pathological mechanisms involved in the breakdown of collagen and protein polysaccharide complexes in human cartilage matrix are being explored in arthritis patients. A series of independent studies are being conducted which are concerned with: Synovial fluid antibodies to proteoglycans and chondrocytes; the organization of proteoglycans; the characterization of the patterns of collagen and proteoglycan biosynthesis and changes in collagen with aging. Most of these studies are being carried out using human articular cartilage or chondrocyte cultures derived from normal and osteoarthritic disease states.

Finally, a study is in progress to develop a diagnostic procedure involving analysis of cartilage lymph aspirated by micropuncture as well as minute whole tissue samples obtained by arthroscopy in patients with osteoarthritis and chondrocalcinosis.

Gout: A project is in progress to delineate the pathogenetic mechanism by which gene product produces aberrations with approaches aimed at therapeutic intervention in the pathological process related to the inherited and metabolic causes of arthritis.

Heritable Disorders: Specific projects directed toward the better understanding of heritable disorders involve the development of a rational basis for enzyme replacement therapy, the correlation of clinical characteristics with biochemical defects and searches for satisfactory methods for the treatment of mucopolysaccharide storage diseases in model systems to determine the nature of the enzyme deficiency.

Systemic Lupus Erythematosus: A study is in progress to characterize the multiple immune systems operative in patients with SLE and assess the relationship of individual immune complex systems to the different forms of the disease. This is coupled with the development and application of sensitive techniques for their isolation and detection. A filterable agent associated with canine SLE has been observed and attempts are being made to associate the agent with clinical findings in the dog with efforts to make applications to human disease. Efforts continue to characterize various antibodies to nuclear antigens, evaluate circulating immune complexes, elucidate factors that determine localization and persistence of preformed immune complexes, and probe the mechanisms of immune complex deposition in kidneys of patients with lupus nephritis. The examination of a large number of factors involved in the pathogenesis of SLE, detailed study of T- and B-cell function, and the

employment of techniques successful in yielding virus in other diseases to tissues obtained from patients with SLE complete the activities highly relevant.

Cartilage: Cartilage studies are concerned with the chemical structure of the carbohydrate chain of keratin sulfate II in the vicinity of the carbohydrate-protein linkage, the elucidation of the antigenic determinants of cartilage proteoglycan and determination of the structural features of heavy chain disease proteins associated with specific biological activities.

Collagen: Various studies in this area are concerned with the isolation, purification, synthesis, inhibition of synthesis, characterization, molecular structure, maturation and degradation of procollagen, collagen, peptides, and other associated constituents. The role of lysyl hydroxylase, the effects of glucocorticoids and comparative studies on collagen in normal and disease states complete the efforts that in general are being supported here.

Connective Tissue: Control mechanisms for glycosaminoglycan production using purified enzymes are being sought. Studies are developing to establish connective tissue cell lines from different sources with efforts to examine the synthesis and structure of connective tissue proteins. Finally there is a large study to define the factors controlling collagen and other connective tissue synthesis and degradation in man with emphasis on inflammatory and metabolic joint disease and metabolic bone diseases.

Ground Substance: Support is being continued for studies in progress to provide needed information related to the isolation, purification, structure, biosynthesis, hormone effects, and quantitative analysis of glycoproteins, and mucopolysaccharides and the involvement of nucleotide oligosaccharides in their biosynthesis.

Synovial Membrane and Fluid: Fine structural analysis of synovial membrane in search of an infective agent in rheumatoid arthritis and the development of a comprehensive picture of the pharmacology and endocrinology of normal and diseased synovium represent the main efforts in this category.

Arthritis, Immune Disturbances: Immune complexes, immunoglobulin peptides, complement sequences, antigen-antibody interactions, the role of suppressor and helper lymphocytes, immune mechanisms and tissue inflammation, lymphocytotoxins, the DNA in soluble immune complexes, anticollagen antibodies, serum agglutinators, immunogenetic correlates of disease, and radioimmune methodology represent the main areas of active interest in this section.

Arthritis, Diagnosis: Use is being made of high resolution auditory sensors and recording devices to characterize sounds made by knee joints during active flexion and extension. Hopefully, pattern classification will differentiate normals from early arthritides.

Muscle and Neuromuscular System: The major emphasis in this area is concerned with the structure, function, contraction and metabolism of skeletal muscle. Specific projects are related to the localization of muscle proteins, molecular structure, cellular calcium compartment analysis, micromolecular

conformation, troponin structure and function, activation mechanisms, regulatory proteins, energy transduction, viscoelastic properties, adaptation to exercise, force-velocity measurements, embryonic growth, fiber heterogeneity, and comparative studies with cardiac and smooth muscle.

## ORTHOPEDICS PROGRAM AREA

The scope of the Orthopedics Program of NIAMDD is broad, including basic and clinical studies of the skeleton under normal and disease conditions. Contained in the program are projects on calcium metabolism and bone formation, fracture healing and fixation, transplantation and preservation of skeletal tissue, prostheses and related biomaterials, biomechanics and bioelectricity, musculoskeletal dynamics and physiology of normal and abnormal gait.

In spite of a total budget of $9.559 million, the number of projects being supported by the orthopedics program has decreased from last year by about 15. In addition to the inflation factor, the increasing direct cost rate is eroding the budget. This decreased funding ability is especially detrimental to the new, bright investigators who are submitting applications in increasing numbers.

The orthopedic training effort is meager. The number of individuals being trained by NIH in orthopedics is about the same as last year. Lack of interest in training support stems partly from the reluctance of clinically trained orthopedists to sign the payback provisions. The orthopedics program supports seven RCDA's only one of which is an orthopedic surgeon. To attract more orthopedists into research the ABSD cluster of NIAMDD has instituted the Clinical Investigator Award for clinicians to obtain research training for academic careers.

The main research areas are as follows:

Prosthetics and Biomaterials: Replacement of diseased joints by prostheses has been the major orthopedics development of the decade. Prosthetic elbows, ankles, shoulders and fingers are being developed and tested. The artificial knee, the second most frequently replaced joint, is not, as yet, as successful as the total hip replacement, and complications appear frequently. As a result, we are supporting an increasing number of applications to investigate the kinematics, biomechanics, and various prostheses for the knee and other joints. At the same time, bioengineers and metallurgists are continuing to investigate better materials for prostheses.

Bone Metabolism: An NIAMDD grantee from Washington University has shown that the inductive potential of bone-bank bone can be improved if the tissue is impregnated with autologous marrow. Studies confirm the presence of osteoprecursor cells in autologous marrow suggesting that composite bone grafts of allogenic bone and autologous marrow cells can support osteoinduction at the level of autografts. As a result, composite grafts may have significant clinical utility in the repair of osseous defects.

In osteogenesis imperfecta, evidence has been presented implicating an abnormal collagen as a source of pathology. Reports of an NIAMDD grantee show there is an abnormal sulfur containing component in OI. Elevated cystine levels suggest it has an amino thio structure.

An NIAMDD grantee using a new sophisticated method of determining bone mass in vivo has demonstrated a significant loss of bone mass in patients with both juvenile and adult-onset diabetes, thus confirming earlier reports linking

diabetes and osteoporosis. Data suggest the hypothesis that loss of skeletal tissue in diabetes reflects the underlying disease and the basic defect may be a decrease in bone formation.

A major finding in the last year is a new protein called osteoclastin which is vitamin K dependent and contains a new amino acid, γ carboxyglutanic acid. It is found largely in mineralized tissues especially those with significant levels of prothrombin and other vitamin K dependent clotting factors, but is also present in a large number of ectopic calcifications such as renal calculi, calcifications of dermatomyositis and scleroderma and calcified plaques of atherosclerotic lesions. This discovery has important implications in the pathophysiology of a wide number of bone diseases.

Fractures and the Healing Process: An NIAMDD grantee in studying the biochemistry of the callus in healing fractures has found a high hexosamine content and $S^{35}$ incorporation within the callus and precallus in the early healing stages. The OH lysine/OH proline ratio within the callus resides between pure bone and cartilage type collagens suggesting the presence of both genetic collagens. Approximately half the collagen synthesis in the callus was type II during the two to four weeks following fracture. The presence of this type of collagen previously identified in the epiphyseal plate provides strong evidence for the concept of endochondral fracture healing.

Several grantees have developed non-invasive methods via resonance, electromagnetics or ultrasonics for measuring the in vivo properties of bone on the rate of healing. The use of electrical stimulation to promote healing of fractures and non-unions continues to be investigated by several researchers. Several difficult non-unions have healed successfully with mild electrical stimulation. Electrical stimulation is also being tested to induce bone ingrowth into porous ceramics with some success. This is an exciting field which holds great potential.

Methods of treating fractures have been under investigation by several grantees. The use of heavy metal plates for internal fixation causes undesirable bone remodeling and osteoporosis. The elastic and tensile properties of bone are being tested to devise better methods of fracture healing.

Cartilage - Structure, Enzymes, and Degeneration: An NIAMDD grantee has discovered that aryl sulfatases A and B levels are elevated in osteoarthritic and chondromalacic articular cartilage. Ascorbic acid is shown to have a dramatic effect on reducing these enzyme levels. Data suggest that ascorbic acid may modify the composition of the extracellular environment of normal and osteoarthritic articular chondrocytes and thus may be useful in osteoarthritis therapy.

Data from another NIAMDD grantee show the presence of collagenase and an inhibitor of collagenase in osteoarthritic cartilage. These findings suggest that degradation of the osteoarthritic surface to the base bone occurs with end-stage osteoarthritis and results from locally synthesized collagenase. This same group in studying the histology and chemical response of articular cartilage over osteonecrotic femoral heads demonstrated limited changes suggesting chondrolysis. This implies an inhibiting effect of loss of integrity of bone

beneath the cartilage in osteoarthritis. Other findings from this same group demonstrate that a low dose of papain promotes healing of articular cartilage defects. It is postulated that the healing process is accelerated due to decreasing proteoglycan content at the wound edges allowing platelet adhesion, fibrin clot formation, fibrinogenesis and chondrification.

In studying the initiation of osteoarthrosis, an NIAMDD grantee has found subchondral bone stiffening in response to repetitive impulse loading which is associated with but does not precede the early metabolic cartilage changes. Loading appears to have an effect on bone stiffness and cartilage metabolism. Although bone stiffness returns to normal after the loading, the cartilage effect does not completely disappear. This experiment negates the concept that bone stiffness clearly precedes cartilage changes following repetitive impulse loading. This same grantee discovered that when degenerative changes in the organic matrix of cartilage occur following massive chondrocyte death, they can progress to produce severe degenerative joint disease in normal viable tibial cartilage.

In studying the antigenicity in joint homograft rejection, an NIAMDD grantee has demonstrated that following transplantation there is a slow release of link protein. Proteoglycan from the donor cartilage matrix and antigens become concentrated in the host synovium. An immune response occurs in these tissues which subsequently is transformed into a pannus and ultimately destroys the homograft.

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In the four research areas just discussed discoveries are being made which slowly add to the knowledge and treatment of related problems. In the area of metabolic bone diseases, however, advances have been few and the number of individuals with these diseases is increasing with our aging population. Calcitonin therapy has shown encouraging results for patients with Paget's disease. Previously combined calcium, vitamin D and fluoride therapy showed promise for osteoporosis treatment, but long-term studies are less promising. Research in the area of osteoporosis poses difficult problems, and new ideas in tackling them are needed. For these reasons in early 1978 NIAMDD is convening a small workshop in osteoporosis. Hopefully from this workshop new ideas will emerge to attack this difficult area.

## DERMATOLOGY PROGRAM AREA

The most recent report by the Senate Committee on Appropriations notes that the Committee is encouraged by the promising results of the new photochemotherapy treatment as outlined in previous reports submitted by the NIAMDD, and described as the first productive new development in the management of psoriasis. As directed by the Committee, the Institute has awarded a contract to support a multi-clinic group of dermatologic investigators to undertake an assessment of both the long-term effectiveness of this new treatment and the long-range safety for the patients so treated. Sixteen university centers will participate in this five-year study.

During the next several months the NIAMDD will develop a task force or special advisory committee to the Institute on psoriasis.

Another special effort in psoriasis supported by the NIAMDD is a contract to establish a coordinating center for a collaborative clinical evaluation of selected topical drugs for psoriasis therapy. The objective of Phase I of the contract was to develop a program for a clinical evaluation of selected chemotherapeutic drugs for the topical treatment of psoriasis. A major effort of Phase I involved the preparation of the Investigative New Drug applications to the Food and Drug Administration for each of thirty drugs to be clinically evaluated. Special consideration was also required by the FDA for specific drug dosage formulation. At the present time, drugs and all accessory materials have been packaged and shipped to five of the seven participating clinical investigators. Twenty-two of the thirty drugs have now been tested in animals. Seven of the preparations were classified as mild irritants. From the experimental test data it appears that the degree of irritation of these drugs was well below that which might be harmful to patients. Testing of the remaining eight drugs is now in progress. Phase II of the contract will concentrate on supervising the multi-institutional clinical testing of the twenty-eight chemotherapeutic drugs approved at this time, and obtaining approval for the remaining two drugs and providing for their eventual entrance into the clinical testing program, and completing the primary irritancy testing in animals. The NIAMDD is pleased with the handling and progress of this contract by the University of Miami, and looks forward to the clinical reports of any potentially effective topical chemotherapeutic agents.

The Senate Appropriations Committee has also advised the Institute of its awareness of the tremendous socio-economic costs which dermatologic diseases inflict on a large segment of the population. Because of the Committee's strong interest in this area, it has recommended that the Institute initiate and support studies to determine the research needs and priorities in the field of dermatology. In response to this mandate, the Institute has received, and is reviewing, a contract proposal from a talented and representative group of dermatologic academicians proposing to carry out and produce this vitally needed information.

In an attempt to stimulate more research activity in vitiligo, the Institute sponsored on March 3 and 4, 1977, a Workshop on Vitiligo.

Vitiligo is a bizarre depigmenting disease of unknown cause that can result in emotional, social, and economic disorder of afflicted patients' lives. It has been estimated that patients with vitiligo may represent one-half to three per cent of the U.S. population. The disease affects races equally although it is obviously more devastating in darkly pigmented individuals. The NIH is currently supporting six grants that are related in some way to vitiligo research. Workshop participants recommended that a common definition of vitiligo be established, that physician education be improved, and that Federal funding for vitiligo research be increased. The Institute is planning to announce a request for applications to foster vitiligo research. It is anticipated that a project proposal for a combined vitiligo diagnostic treatment and research center from Yale University and Howard University will be submitted.

Brief highlights of current supported research sponsored by the Dermatology Program Area are as follows:

Epidermolysis bullosa is a disabling and sometimes lethal blistering disease of children and adults. Grantees have reported that there is strong evidence available that human skin collagenase is present in the skin lesions of patients with epidermolysis bullosa. If this is confirmed, it could be determined that human skin collagenase plays a primary role in the pathogenesis of this disease.

Grantees at the University of Michigan are in the process of developing a system to detect "tumor-associated protease activity" which is considered to be an early event in malignant transformation of cells.

Acceleration of research in the new field of photobiology is being conducted by Institute grantees. These include investigation of photosensitivity which includes phototoxicity, photo-allergy, polymorphous light eruption, solar urticaria, the porphyrias, and other light-induced diseases. They are also examining the long-term ultraviolet-induced effects which include aging, carcinogenesis, and the effects of ozone depletion. In the area of photoprotection, they are examining the effects of beta carotene, phytoene, anti-prostaglandins, and topical sun screens. Photochemotherapy as relating to psoriasis, vitiligo, and herpes simplex is also being studied. In addition to the five-year contract to follow psoriasis patients treated with photochemotherapy, there is a project under way to confirm or negate recent reports that the combination of psoralens plus long-wave ultraviolet light can result in chromosome damage.

Grantees are utilizing newer immunologic techniques to examine contact dermatitis and related disease states. At first thought these conditions were believed to be pure delayed hypersensitivity or T-cell mediated, but are now thought to have involvement of the bone marrow-derived (B-cell mediated) system as well. Positive immunofluorescent patterns in patients with patch test contact dermatitis have been found. Contemplated research includes looking further into the techniques of identifying the cells involved in contact dermatitis.

Grantees continue their attempts to elucidate the pathogenic factors in acne vulgaris, so that preventive measures and more effective therapy can be formulated. Efforts are concentrated on the metabolism of sebaceous lipids because they are widely considered to be involved in both the initiation and maintenance of inflammatory acne. However, in addition to the measurements of lipid composition in acne patients, there are concomitant measurements of the microflora in patients being treated with antibiotics. The determination of the reason why certain patients do not respond to systemic antibiotic therapy remains an important goal of grantees.

Pathomechanisms involved in the development of organized epithelioid cell granulomas such as occur in leprosy, tuberculosis, and sarcoidosis are the subject of grantee investigation. An important but highly controversial issue centers on the role of cell-mediated immunity in granuloma formation. Passive transfer experiments by grantees completed in the past year seem to support the idea that transfer factor is incapable of regularly transferring granulomatous hypersensitivity.

As the primary cell of the skin, the keratinocyte moves outward through the epidermis. It ceases dividing and engages in synthetic activity directed toward the production of keratin and presumably other matrix proteins and phospholipids. Grantees report that this alteration in cell function probably reflects a changing pattern of genetic transcription. They are attempting to determine if the processes of division and keratinization are influenced by cyclic AMP, and that the effect of cyclic AMP on transcription may be mediated by nuclear acidic proteins and/or histones.

## ASSOCIATE DIRECTOR'S ANNUAL REPORT

### National Commission on Digestive Diseases

A National Commission on Digestive Diseases was established under the October, 1976 Amendments to the Arthritis, Diabetes, and Digestive Diseases Act.

The Commission will: conduct a comprehensive study of the present state of knowledge of the incidence, duration and morbidity of, and mortality rates resulting from digestive diseases and of the social and economic impact of these diseases; evaluate the public and private facilities and resources (including trained personnel), for the diagnosis, prevention, and treatment of, and research in such diseases; and identify programs (including biological, behavioral, nutritional, environmental, and social programs), to improve the management of digestive diseases.

Based on the study described above, the Commission shall develop and recommend a long range plan for the use of national resources to effectively deal with digestive diseases. The plan shall provide for: (1) evaluation of new and improved methods of screening for digestive diseases; (2) development of preventive measures; (3) development of criteria for the diagnosis and the clinical management and control of digestive diseases; (4) development of coordinated health care systems; (5) education and training of scientists, clinicians, educators, and allied health professionals; (6) establishment of a standardized nomenclature of all digestive diseases for use in basic and clinical research and to facilitate collaborative studies; (7) the conduct and direction of field studies and clinical trials; (8) research studies; and, (9) establishment of a system of periodic surveillance of the research potential and research needs in digestive diseases corresponding with the recently completed survey organized by the National Institute of Arthritis, Metabolism, and Digestve Diseases.

The Commission includes 17 members appointed by the Secretary of Health, Education, and Welfare, and 9 ex officio members from government institutions that are administering health programs and activities related to digestive diseases.

The Commission held its first meeting April 11th, in Bethesda, Maryland. It heard a review of some relevant previous activities. It then selected as its chairman, Dr. Eugene Jacobson, Associate Dean of the College of Medicine, University of Cincinnati, Cincinnati, Ohio.

### Digestive Diseases Coordinating Committee

A Digestive Diseases Coordinating Committee was established to coordinate activities in digestive diseases in the agencies of the Federal government dealing with digestive diseases. The members have exchanged information on the activities of their agencies, and are preparing material for the Commission.

## Workshops

A workshop on Nutritional Fiber, supported through the Nutrition Coordinating Committee of the National Institutes of Health, was held in Bethesda, March 27-29. It brought experts together in study sessions on: measurement of nutritional fiber; interaction between fiber and intestinal bacteria; fiber and the digestive tract; fiber and cardiovascular disease; fiber and cancer; and fiber and obesity.

A workshop on the training of gastroenterologists in cancer was held in Bethesda, November 19, 1976. A group of experts discussed: (1) the responsibility of the Gastroenterologist in Cancer; (2) the training required to prepare a gastroenterologist for oncology; and (3) the mechanisms and resources available for providing the training. Guidelines for training in this area will now be prepared, along the lines discussed during the conference.

Digestive Diseases Program Area
(1976-1977)

The Digestive Diseases Program Area is responsible for the extramural support of grants awards and contracts pertaining to certain organ systems of the gastrointestinal tract. These include the esophagus, stomach and duodenum, and small and large intestine. Also included in this program area are studies of the cause, prevention, diagnosis, and treatment of diseases of these organ systems such as achalasia, esophageal reflux, ulcer disease, colonic and inflammatory bowel diseases, and the psycho-social aspects of digestive diseases. Finally, support is provided for research development, and evaluation of techniques, methods or approaches in the use of endoscopic and other instrumentation for the diagnosis, treatment, and investigation of relevant digestive diseases.

A. Research Activity Supported By Grants

During FY 1976 much of the activity supported by research grants was concerned with the basic aspects of the physiology and biochemistry of disease activities. Of these, many have clinical implications although the studies were performed in animal models. Several of the projects were clinical investigations whose results are directly applicable to human disease. Of the considerable amount of data and information reported by the grantees during the past year, only a few examples will be cited as representative of the progress that has been made in the Digestive Diseases Program Area.

1. Recent Advances in Viral Gastroenteritis

Viral gastroenteritis is a common disease of short duration characterized by nausea, vomiting, abdominal cramps, diarrhea, and other varying symptoms. It occurs in both epidemic and endemic forms, and although rarely fatal in developed countries, it is the second leading cause of illness in the United States. Consequently, the economic consequences of lost work hours due to gastroenteritis are considerable. In the chronically malnourished, the elderly, and in debilitated populations, gastroenteritis may also be a significant cause of death.

Recent application of new virological techniques and the use of electron microscopy to identify viral particles and their interaction with antisera have resulted in the identification and partial characterization of two types of viral gastroenteritis agents; parvovirus-like agents which appear primarily responsible for disease in adults and older children, and reovirus-like agents which appear to be a major cause of viral diarrhea in infants and young children. New information is now available regarding

the clinical features, the histopathology and some aspects of the pathophysiology of viral enteritis. Nevertheless, there is a need for considerable more research activity. The mode by which the viruses which cause gastroenteritis gain access to intestinal mucosal cells is unknown. Development of practical methods for cultivation of the viruses which cause gastroenteritis are needed. The factors that determine host resistance to viral enteritis remain unknown. The interrelationship of viral gastroenteritis to chronic intestinal diseases requires extensive study. Finally, the feasibility of immunization against gastroenteritis should be explored.

2. Recent Advances in Peptic Ulcer Disease

a) Ulcer disease afflicted 4 million people in the United States in 1975 with an overall prevalence rate of 1900 per hundred thousand, and accounted for eight thousand deaths. Few new or revised concepts concerning the pathogenesis of peptic ulcer disease have evolved during the past few years. On the other hand, numerous well designed studies are providing data to establish the merits of the current concepts. Duodenal ulcers have long been regarded as a disease of excessive acid secretion and gastric ulcer as a defect in gastric mucosal resistance. More specifically, three factors may play a role in the pathophysiology of duodenal ulcer disease: increased capacity to secrete acid, increased responsiveness to stimuli of acid secretion, and defective inhibition of acid secretion. Clinical investigations have now established that duodenal ulcer patients have not only a greater than normal maximal secretory capacity but, also secrete at higher than normal rates with submaximal stimuli. Also, a dual defect is present during meal-stimulated secretion. Impaired antral inhibition of gastrin release contributes to excessive acid secretion and rapid gastric emptying reduces the buffering effect of the meal itself. The result is a large load to the duodenum where inhibitory mechanisms and pancreatic secretion are not sufficient to control the acid secretion.

b) Two new classes of pharmacologic agents have shown great potential in controlling gastric acid secretion which is primarily responsible for the formation of duodenal ulcers. Cimetidine, one of a new class of histamine $H_2$ receptor antagonists and $PGE_2$, a member of the ubiquitous biogenic compounds classified as prostaglandins. The event of cimetidine was greeted with considerable enthusiasm with some speculation that the therapy of peptic ulcer disease would be completely changed or at least replace the liquid neutralizing antacids in the standard ulcer

regimen. Cimetidine and the antacids reduce the acid entering the duodenum by completely different mechanisms. The $H_2$ receptor antagonist inhibits the secretion of gastric acid whereas the antacids neutralize the acid that has already been formed and delivered to the duodenum. Cimetidine is more effective in the fasting state and its inhibitory action on nocturnal acid may be marked and prolonged. Nevertheless, clinical studies are demonstrating that with the proper dosages, both treatment modalities gave comparable results. It is recognized that patient compliance for liquid antacids is generally poor because of the resulting diarrhea or constipation and poor palatability. In several instances, ulceration reoccurs shortly after cessation of therapy, particularly after usage of cimetidine. Nevertheless, the $H_2$ antagonists may be the drug of choice because of convenience and enhancement of efficacy; however, long term maintenance therapy may prove expensive, and some patients may not tolerate the new drugs. Liquid antacids will still provide a rational alternative therapy for duodenal ulcer disease during daytime, and cimetidine the choice for noctural inhibition of gastric acid secretion. Some combination mode of therapy will evolve to accommodate most patients and for long term usage in the prevention of reoccurrence of duodenal ulcers.

3. Recent Advances in Inflammatory Bowel Disease

a) Inflammatory bowel disease remains a mystery for thousands of persons afflicted with colitis or Crohn's disease. The pathogenesis of these diseases is not understood. Therapeutic modalities are limited and empirical. Some recent evidence strongly suggests that inflammatory bowel diseases may result from immunologic disorders while other types of colitis and enteritis may be a result of infection by as yet unidentified pathogenic organisms. Research in this field has been sorely hampered by the absence of an appropriate animal model for the disease. Recent studies have reported four such animal models thay may be useful for future studies on the etiology of certain inflammatory bowel disease and certainly for research on the therapy or prevention of these pathological conditions. One recommendation is an inbred strain of boxer dogs with spontaneous occurrence of ulcerative colitis, histologically indistinguishable from the human disease. Two other animal models of colonic disease are the clindamycin-associated colitis in hamsters and the carrageenan guinea pig model of ulcerative colitis. Of the two, the latter model shows more promise. The histological changes in animals given carrageenan appear to be identical to those seen with ulcerative colitis in humans. Additionally, these lesions have been noted in virtually all animal species tested including

primates. This model affords a unique opportunity to trace the entire evolution of disease from its inception to the final clinical expression.

A fourth animal model has been developed from the observation that many patients with ulcerative colitis have been reported to have lymphocytes which are cytoxic for colonic cells in vitro. This would suggest that a cellular immune response may be part of the pathogenesis of ulcerative colitis. A model of ulcerative colitis related to cellular immunity has been produced by sensitization of the colon of rabbits with dinitrochlorobenzene. Histologic changes in the colon of the rabbit were produced that were compatible with human ulcerative colitis with lymphocytes that were sensitive to colonic antigen.

b) The medical management of Crohn's disease, one of the inflammatory bowel diseases, is currently largely empirical and often unpredictable, depending a great deal on the acuteness or chronicity of the disease. There has been a significant increase in the incidence of the disease among young people, particularly adolescents. Adolescents with Crohn's disease reportedly have a high morbidity and therapeutic results are often temporary and unpredictable. Frequent relapses, disease progression, and a degree of incapacitation are almost inevitable. There are few published guidelines as to the therapy of this disease and in general supportive measures are used such as diet, rest, sedation, and antidiarrheal medications. Salicylazosulfapyridine and adrenal corticosteroids are used both for therapy of relapses and for long term administration. The therapy of Crohn's disease in adolescence must balance the natural disease history of growth suppression, debilitation, and progression against possible drug related adverse effects on growth and development. A limited clinical study has been reported demonstrating successful efforts in providing sympto-matic control and normal rate of growth albeit in patients with primarily ileal disease. The rationale of the therapy was to suppress disease activity with the minimum dosage of adrenal corticosteroid on an alternate day basis in order to avoid linear growth suppression. Salicyazosulfapyridine or tetracycline were used as adjunctive therapy throughout the course of illness. Although successful therapy was achieved, it should be emphasized that this was a preliminary trial and not controlled in the classical approach; consequently, an adequately controlled trial of the concepts evolved from this reported study is highly recommended.

4. Recent Advances For Gastrointestinal Hormones

Research activity and progress in the area of gastrointestinal hormones during this reporting period has been so abundant and productive that it would be difficult to select any

one or more studies to be representative of the total progress that has been achieved. Rather, it would be more significant to present an overview or state-of-the-art of our current knowledge of the gastrointestinal hormones and related substances.

The gastrointestinal hormones and related substances can be classified in three distinctive groups. The first class include those substances that have been chemically defined. The amino acid sequences of 7 peptides derived from the gut endocrine-paracrine cells are known: gastrin, secretin, cholecyst-okinin (CCK), gastric inhibitory peptide (GIP), vasoactive intestinal peptide (VIP), motilin, and substance P. Chymodenin, claimed to stimulate selectively secretion of chymotrypsin by the pancreas, has been isolated but not yet sequenced. The second class include the immunoreactive substances. Extracts of gastric and intestinal mucosa contain substances that react with antibodies to glucagon, somatostatin, and bombesin. The chemical nature of the immunoreactive substances have not yet been determined but they can be presumed to be related to the antigen used to raise the antibody with which they crossreact. The third major class include ten substances that have been obtained from crude extracts of mucosa with a variety of actions on gastrointestinal and other targets. In most instances the biological actions are one shared with known peptides so until the active principles are isolated and chemically identified there will be uncertainty as to whether these actions are attributable to known substances or new ones. These crude extracts include such substances as: bulbogastrone, duocrinin, enterocrinin, enterogastrone, enter-oxyntin, gastrone, gastrozymin, incretin, pancreotone, villikinin. Many of the gastrointestinal hormones and related substances have a variety of actions on secretion and motility of the digestive tract. Several of them such as gastrin, cholecystokinin, secretin, and gastrin inhibitory peptide have been shown to be released under conditions that would link them to physiological events. Feeding increases the blood levels of gastrin, CCK, secretin, GIP, and glucagon-like immunoreactivity (GLI). Blood levels of VIP and motilin do not change in response to ordinary feeding. It has been found that instilling strong alkali or strong acid into the duodenum raises blood motilin levels.

Substance P, bombesin-like immunoreactivity, and somatostatin-like immunoreactivity have not yet been demonstrated to be present in blood and no mechanism for their release from gut has been described. On this basis, only 4 peptides may be regarded as hormones, e.g. gastrin, CCK, secretin, and GIP. Six other peptides such as VIP,

motilin, substance P. glucagon-like, bombesin-like and somatostatin-like identified by immunoreactivity must be regarded as "candidate" hormones are recognized. Gastrinomas of the pancreas, duodenum, or rarely the stomach produce the Zollinger-Ellison syndrome (hyperplasia of gastric mucosa, hypersecretion of acid, severe peptic ulcer disease). Tumors that secrete VIP (vipomas) may arise from pancreas or nerves and cause the pancreatic cholera syndrome (severe watery diarrhea). Many diseases cause secondary changes in gut hormones, e.g. patients with pernicious anemia have antral G cell hyperplasia and hypergastrinemia.

B. Research Activities Supported by Contract

1. National Cooperative Crohn's Disease Study
   Contract No. NO1-AM-2-2210
   University of Colorado, Denver, Colorado
   Total Duration - 6 Years Total Costs - $1,877,769

The National Cooperative Crohn's Disease Study (NCCDS) has completed the first two phases of the study that was initiated in 1972 to determine the efficacy of three drug modalities in the control of active symptomatic Crohn's disease during four months of high dose treatment; also, to determine the efficacy of these drugs, e.g. prednisone, sulfasalazine, and azathioprine for the prevention of flare-up or recurrence of quiescent disease by low dose treatment.

The NCCDS has studied 584 patients with Crohn's disease at 14 centers in a randomized, prospective, double blind study. 300 patients with active symptomatic disease were followed for 17 weeks. Response to prednisone or sulfasalazine was better than placebo. Response to azathioprine did not differ from placebo. In patients on steroids at randomization, substitution of sulfasalazine was no better than substitution of placebo; however, continuation of prednisone was superior to placebo. For those patients who responded favorably to any one of the study drugs, they continued on the same drug therapy but at a lower dose in order to determine whether flare up or recurrence could be prevented during the ensuing one or two years. Response to any drug was no different than to placebo. These same results were obtained in those patients with quiescent disease at the time of entry into the study. Moreover, clinical or x-ray deterioration had occurred in 32% at one year and 69% of the patients at two years.

A third phase of the Crohn's Disease Study was initiated this past reporting period with a "Trial of Adjunctive Therapy". It was planned to be a limited study of fifteen months entailing a study of approximately 100 patients with active symptomatic disease. The objectives were to 1) compare the efficacy of

prednisone alone versus the combination of prednisone and sulfasalazine in the treatment of acute symptomatic Crohn's disease; 2) to determine the "prednisone sparing" effect of sulfasalazine with those patients who have responded successfully to therapy in accordance with (1); 3) if sulfasalazine fails to demonstrate a "prednisone sparing" effect, to determine whether it will sustain a prednisone-induced remission.

These studies have been conducted under the direction of Drs. John Singleton and Fred Kern, Jr. of the University of Colorado

2. Research and Development Studies in Gastrointestinal Endoscopy

a. Contract NO1-AM-5-2211
Title: Endoscopic Control of Continued Massive Gastric and Duodenal Bleeding in a General Hospital Population
Institution: University of Washington, Seattle, Washington
Duration: Two Years
Contract Amount: $273,397

b. Contract: NO1-AM-5-2213
Title: 1. Endoscopic Retrograde Cholangio-pancreatography
2. Endoscopic Electrosurgery for Control of Upper G. I. Hemmorrhage
Institution: University of Minnesota, Minneapolis, Minn.
Duration: Two years
Contract Amount: $138,094

These contract studies are being supported to study the control of gastrointestinal bleeding by endoscopic systems involving laser coagulation, electrocoagulation, suture clips, and tissue glues. To date, significant progress has been made. Biocompatible tissue glues and suture clips have failed to stop severe bleeding in the ulcer model that was developed during the early phases of the contract. Three modalities have been found to be most promising for safe and effective control of massive upper gastrointestinal bleeding. These include the argon laser, the heater probe, and electrocoagulation. The argon laser used in conjunction with the endoscope and with pressurized carbon dioxide has proven to be fail-safe with a wide margin of safety for the patient and the operator. This modality is essentially ready for controlled prospective trials in massively bleeding patients. The other two methods require additional study and development together with appropriate animal testing before controlled trials in one or more medical centers can be initiated.

The studies at the University of Washington are under the direction of Drs. Fred Silverstein and Cyrus Rubin. The studies at the University of Minnesota are under the direction of Drs. Jack Vennes and Stephen Silvis.

Liver Diseases Program Area
(1976 - 1977)

During the past year the Liver Diseases Program Area has developed plans for 4 major workshops, and initiated the major 900 patient controlled clinical trial on the use of chenodeoxycholic acid for gallstone dissolution. The major area of support remains the individual research grant program supporting activities concerned with the physiology, biochemistry and morphology of disease entities involving the gallbladder and biliary tract, the liver and the exocrine pancreas. Some of the diseases within this area include gallstones, biliary atresia, cholestasis in adults, acute and chronic liver diseases of both inflammatory and metabolic origin, acute and chronic pancreatitis. Twelve research training grants are being supported. The research fellowship program and the career program continue to be viable but are not up to previous years strength.

A. Research Grants

Of the many significant advances made during the past year in the Liver Diseases Program Area by Institute -supported grantees, only a few examples will be cited. These deal with the following topics:

1. Insulin and Glucagon protection in hepatic failure
2. Formation of pancreatic calculi
3. Rate processes involved in gallstone formation
4. Fasting hyperbilirubinemia
5. Blood Marker for alcoholism
6. Biliverdin IX a: A new probe of hepatic bilirubin metabolism

1. The Effect of Insulin and Glucagon on Fulminant Murine Hepatitis

The most serious complication of viral hepatitis is fulminant hepatic failure in which the mortality of those patients in coma is about 80-90%. Attempts to sustain life by supportive measures until spontaneous regeneration can occur have so far been unsuccessful. However, a new approach to enhance liver regeneration by the synergistic action of two well-known pancreatic hormones-insulin and glucagon- has met with spectacular success in an animal model.

When young mice are injected with a large dose of a particular mouse-hepatitis virus, 100% of the animals die within 4 days. Grantees at Harvard University and at the Massachusetts General Hospital found that they could save 40% of the infected mice if they immediately started an infusion of insulin-glucagon. Even delaying treatment for 24 hours was able to lead to recovery of 15% of the mice. In contrast to the combined therapy, insulin alone had only a modest effect and glucagon

none. Details of the most effective ratios of insulin: glucagon for the greatest synergistic effect was also studied. In the dual-hormone-treated group, 24 hours after initiation of treatment, the liver cell necrosis was only half as extensive and by 6 days the liver histology was almost normal.

The next step in this exciting research should be to determine whether this combination can also beneficially influence the clinical course of acute liver-cell injury in man.

2. The Physical Chemistry of Pancreatic Calcification and Stone Formation

Calcification is an important and frequent complication of chronic pancreatitis and hypercalcemia is a common and potentially dangerous complication of acute pancreatitis. Pancreatic stones are about 95% calcium carbonate and a possible mechanism for their formation has been postulated by a grantee at the Medical College of Virginia. The formation appears to be determined by the limited solubility of the calcium ion in solution which is in turn determined by the pH, bicarbonate and carbonate ions of the pancreatic secretion. Therefore, construction of triangular coordinate plots, such as have proved extremely valuable in defining the area of cholesterol solubility in gallstone formation, is proving extremely valuable in understanding another situation in which the saturation index is exceeded and stone formation begins. Whether this information can be used to prevent the formation or to dissolve the already formed stone is a potential hope for the future.

3. Rate Processes Involved in Gallstone Formation

Although a lot of research has recently focused on the dissolution of cholesterol gallstones, little effort has been directed toward the understanding of how crystal formation begins in the bile and nucleation, growth, and aggregation occur in bile. Various substances such as bacteria, mucus, and proteins have been variously thought to be the substances around which crystals form (i.e., nucleators). Physical chemists at Carnegie-Mellon University have shown that no such components are necessary and that cholesterol crystals can grow in model bile solutions containing only the biliary lipids - bile salt, lecithin and cholesterol.

At cholesterol concentrations below that previously believed to be the lower limit of supersaturation, submicroscopic growth stops before microscopically visible sized crystals are reached.

When such a "subsaturated" solution is mixed with a highly supersaturated solution containing large rapidly growing crystals, the large crystals persist and continue to grow even though the bile is no longer supersaturated because of the dilution.

This important basic research has also looked at crystal form and the investigators have been able to produce two types of crystals - screw and twin cholesterol crystals from model bile. The twinned crystals resemble photographs of sectioned gallstones and are the familiar pseudorhombic crystals sometimes seen in the bile of patients with gallstones.

4. Hyperbilirubinemia of fasting

Although several investigators have demonstrated that a high serum bilirubin may result from a prolonged fast, the mechanism for this effect remains a mystery. Fasting hyperbilirubinemia is an important problem in the neonate and the commonly used nutrient solutions such as glucose or protein have no effect. However, feeding even small amounts of fat, as low as 10% of the caloric requirement, rapidly reverses the bilirubinemia in experimental animals (Gunn rats).

Now a pediatric gastroenterologist at the University of Calif. San Francisco, has shown in the rat model that if he can prevent lipolysis from occuring by the administration of inhibitors such as nicotinic acid or insulin, he can also prevent or reverse the hyperbilirubinemia. This may be because the pigment released during fasting is derived from depot fat. He postulated that since the feeding of fat in even very small amounts triggers the release of insulin, this may be the mechanism for the beneficial effect of fat feeding on hyperbilirubinemia.

5. A Biochemical Marker of Alcoholism

Alcoholics are noteably unreliable in reporting their alcohol consumption or the severity of their disease. Blood alcohol levels, or blood enzyme changes are not reliable and do not distinguish between long term and short-term alcohol consumption. Now grantees jointly supported by the NIAAA and the NIAMDD have demonstrated good correlation between plasma constituents and the degree of alcoholism as separately assessed by three separate medical and psychological criteria. The investigators at the Mt. Sinai School of Medicine, N. Y. and the Bronx, V.A. admit to puzzlement about the mechanism. The finding of an increased ratio of $\alpha$-amino-$\gamma$- butyric acid to leucine (a branched chain amino acid) in chronic alcoholism is entirely empirical. However, it may be a useful test in the detection and management of alcoholism.

6. Biliverdin IXa ; A New Probe of Hepatic Bilirubin Metabolism
Being able to follow the breakdown of hemoglobin and its excretion as bile pigments into the bile has proved to be a very complex undertaking. An organic chemist who is part of the Liver Center at the University of California, San Francisco, recently suceeded in a very difficult synthesis, the production of an intermediate of heme catabolism (biliverdin IXa). This compound can be converted to bilirubin IXa by a reductase found in mammalian tissue. However, the in vivo fate of biliverdin IXa had not previously been studied until its recent synthesis. These investigators have now shown that the biliverdin is rapidly cleared from the blood by uptake by the liver. A small portion is excreted directly into bile but most is reduced in the liver to bilirubin, which is then returned to plasma in its unconjugated form. This bilirubin is subsequently taken up by the liver, conjugated and excreted into the bile. The bilirubin so formed seems to be kept in a segregated compartment in the liver from that taken up directly from the plasma and may be a useful tool to investigate hepatic bilirubin transport and compartmentalization.

B. Contracts

National Cooperative Gallstone Study NO1-AM-3-2216
Cedars-Sinai Medical Center, Los Angeles, California
$7,215,540 for 5 year period.

The Contractor, acting as the Coordinating Center is charged with the execution of a clinical trial to determine the safety and efficacy of chenodeoxycholic acid (an experimental drug) in dissolving cholesterol gallstones. The main 900 patient clinical trial began in September, 1976. This followed the successful completion of the protocol development and planning phase (including extensive animal toxicity studies) and the accession of patients to a 100 patient preliminary study. Although there were various time delays in the preliminary phases due to various requirements of the FDA before initiating the main study, the 900 patient clinical trial is proceeding on schedule. Each of the 10 treatment centers expects to accession 90 patients into the study over an 18 month period. Each patient will receive therapy (randomized, double-blind) for a 2 year period or until gallstone dissolution occurs. The expected termination date of the study is November, 1980. Various safety monitoring procedures are incorporated into the study to insure that if any significant toxicity occurs or if an unfavorable benefit/risk ratio occurs, the study can be terminated.

C. Workshop Plans

Various areas of research in hepatic and biliary tract diseases were considered appropriate for stimulation and/or clarification of the state of art. The four workshops to be conducted under NIAMDD sponsorship are all areas which also have relevance to other Institutes and are to be jointly sponsored. The dates, title of workshop, chairmen and sponsors are as follows:

| | |
|---|---|
| Feb. 7- 9, 1977 | Fulminant Hepatic Failure<br>Drs. Paul Berk & Hans Popper<br>NIAMDD and FIC |
| Feb. 28, March 1, 1977 | Collagen Metabolism in the Liver<br>Drs. Karl Piez and Hans Popper<br>NIAMDD and NIAAA |
| March 21 -23, 1977 | Biliary Atresia<br>Dr. Norman Javitt<br>NIAMDD and FIC |
| May 9 - 11, 1977 | Hepatotrophic Factors<br>Dr. N. McIntyre<br>NIAMDD and Ciba Foundation |

ANNUAL REPORT FY '77
08 Nutrition Program Area

The Nutrition Program, NIAMDD-EP is responsible for supporting projects in nutrition research, career development and training in the areas of basic and clinical nutrition. The ultimate goals are to prevent disease and promote optimal health, thus improving quality of life. It is recognized that some nutrition research is funded by other NIAMDD programs where nutrition is a secondary but related factor.

Funding Accomplishments

The NIAMDD supported approximately 24% of the nutrition research and training administered by NIH. The NIAMDD Nutrition Program has given special emphasis to the following research areas: obesity, parenteral nutrition, nutritional anemias, trace element nutrition, dietary "fibers", mechanism of action of nutrients, nutrient interactions and factors that modify nutritional requirements. The NIAMDD also administers the US-Japan Malnutrition Panel which is concerned with nutrition research needed to solve health problems of South East Asia.

The total budget for the fifteen month period, July 1, 1976 - September 30, 1977, was 11.588 million (9.606 million for FY 1977 plus 1.982 million for the transitional quarter of July 1 - September 30, 1976). The FY 77 budget included 0.8 million earmarked funds for nutrition research. In addition, a total of 1.296 million from the diabetes program budget has been used to fund 12 diabetes-related nutrition research grants and it is anticipated that six additional applications will be funded from this source before the end of FY 77.

Including those funded by Diabetes Program monies, the total number (145) of nutrition research grants in FY 77 remains about the same as in FY 76 (153) but shows a marked decrease when compared to the number (209) funded ten years ago in FY 66. However, the number of research grants funded from only Nutrition Program monies dropped by 16% to 128. The trend of funding a fewer number of research projects with increases in budget is a recognized consequence of higher cost of research and escalation in rates of indirect costs charged by institutions. Three program projects continue to be funded, one of which was renewed in FY 76.

Ninty competing research applications were approved by Council; only 33 of these or 37% will be funded.

Advisory Group Recommendations

A Nutrition Advisory Group was convened January 17, 1977 to review the nutrition program activities and advise on priority areas that should receive greater support in view of present research needs and opportunities, as well

as budgetary constraints. The report emphasized the need to continue efforts in refining our knowledge of human requirements for nutrients and supporting basic research. In addition, it is suggested that emphasis be given to five priority areas:

1. coordinating studies on causation and treatment of obesity, including the establishment of multipurpose centers.
2. take a lead in promoting basic studies on principles and practice of supportive nutrition for patients with illnesses leading to overt or sub-clinical states of malnutrition.
3. pursue more vigorously the prevention of anemia due to iron deficiency, and search for improved methodologies.
4. study more extensively the potentially important role of new trace elements, human requirements, and manifestations of deficiency and toxicity states.
5. probe the nutritional implication of the content and source of "fiber" in the diet.

Training Efforts

The Nutrition Program in NIAMDD is the main source for funding development of nutrition research manpower. The National Research Service Act which authorizes individual fellowships and institutional training programs supports postdoctoral training and a limited number of predoctoral positions. It is recognized that nutrition differs from other disciplines. To bring promising candidates into the field, students in biochemistry, medicine and other fields of science are encouraged to pursue nutrition research interests by fellowship and career development programs. Presently support is being given for 13 individual fellowships (NSRA-F32); 2 career awards (K06) - a life time award to pursue full time research activities; 6 career development awards (K04); 3 academic career awards (K07); 1 clinical investigator award (K08) and 6 institutionsl training awards (T32). All the "old" training programs (T01) terminated June 1977. It is anticipated that future budgets will allow an increase in the number of awards to continue training manpower for nutrition investigation. One priority area for the future is to focus on clinical and basic nutrition research and training programs in medical schools.

Research Trends and Highlights

1. Obesity

Obesity remains a major health problem in the United States and the health of millions of Americans is impaired by calorie excesses and an imbalance of nutrients. This challenge is being met by funding a variety of research projects concerned with obesity, and appetite. A major undertaking is the support of one obesity center at St. Luke's Hospital affiliated with Columbia University's College of Physicians and Surgeons, New York City for the multidisciplinary investigation of obesity. Now in its fourth year, the center is emerging. The core research deals with human obesity as characterized by food-related and metabolic behavior, satiety signals,

management of the obese patient, research of carbohydrate, fats and protein as well as many other parameters.

In addition to the center grant, 16 research grants deal with obesity and 15 with physiological appetite and behavior modifications.

Total funding in this area amounted to 1.9 million of direct costs or approximately one-fourth of nutrition research support. However, less than 1 million comes from the Nutrition Program budget, with the remainder being funded with diabetes program monies.

An additional 1.2 million would be required in FY 79 to implement a clinical study and treatment component at four locations involved in obesity research, as recommended by the Nutrition Advisory Group.

2. Supportive Nutrition for Hospital Patients

Despite the increased awareness of the need for better nutritional support of patients in hospital, extended-care facilities and other situations, far too little research attention is being given this widespread problem. The Advisory Group recommended that NIAMDD give high priority to developing more complete basic information about the nutrient requirements of patients in the presence of various disease states. Among the specific needs is an improved system and better methodology to assess nutritional status in the laboratories. Parenteral nutrition, for example, would be facilitated by a battery of laboratory tests that could be performed on small amounts of blood, urine, or serum. This will receive priority attention and a special workshop is being planned in cooperation with the American Medical Association on improved methodology for rapidzassessment of nutritional status.

During FY 77, four research grants were supported, totaling 285 thousand in direct costs or 4.2% of the budget. Plans are being developed for a new mechanism to stimulate "nutrition support units" in hospitals associated with medical schools on a regional basis. Development and funding will be done in collaboration with other institutes. It is estimated that such a new program would require approximately 3 million per year at the start but would become cost effective.

3. Dietary "Fiber"

Epidemiological data suggests that low intakes of dietary "fiber" may be correlated with the increased incidence of a number of diseases including cardiovascular disease, cancer of the large bowel, irritable bowel syndrome, diabetes, appendicitis, diverticular disease and even obesity. Accordingly, research on the role of dietary "fiber" in health maintenance and treatment is assigned a high priority. Emphasis will be placed on developing knowledge of possible mechanisms of action and on the development of routine methodologies to quantitate the various components of dietary "fiber". Possible negative effects will also receive attention, as the ability of some fiber components to chelate trace minerals rendering them unavailable for absorption.

An NIH wide dietary "fiber" workshop was co-sponsored in March 1977 by NIAMDD in collaboration with other institutes through the NIH Nutrition Coordinating Committee. The nutrition program has received four research grants on dietary fiber three of which will be funded in FY 77, totaling 153 thousand direct costs. The amount required to support research in the "fiber" area can be expected to increase to 800 thousand by FY 79.

4. Newly Recognized Essential and Trace Minerals

The area of trace element requirements and their possible role in etiology of chronic, adaptive diseases has become one of increasing concern with the recognition of the essentiality of an ever increasing number of trace elements. Animal studies have established the essentiality of tin, vanadium, silicon, nickel and fluoride for growth. Additional data from K. Schwarz (UCLA) suggests that arsenic and cadmium also may be required under certain conditions. The role of zinc, chromium and selenium in metabolism of animals and man is now recognized.

Although it is well establish that iron deficiency anemia is widespread in the USA and in most other countries, only recently have the relatively low intakes of zinc and copper been recognized as not uncommon. This greatly expands our concern about the adequacy and proper balance of trace minerals and effects on health. For example, it would be reasonable to hypothesize that suboptimal intakes of such elements could result in a depraved appetite, and a tendency to overeat. The practice of "pica" does occur in up to 20% of certain population sub-groups. The possible relationship of sub-optimal intakes of trace minerals, modified eating behavior and gradual increases in body weight and obesity has only recently been recognized and requires study.

Information on requirement and balance of trace elements is particularly important in view of the narrow range of safe intakes of many trace elements and the marked effect of specific imbalances. For these reasons this is considered to be a priority research area. Emphasis is being placed on the metabolic roles, interactions with other minerals, factors which affect bioavailability and the need for rapid, more reliable analytical methods for many of the trace minerals.

Due to the widespread occurrence of iron deficiency anemia which lends itself to dietary supplementation, field studies are needed to evaluate the health significance of nutritional anemias with emphasis on work performance and susceptibility to infection. Recent advances in methodologies for measuring iron stores and the bioavailability of iron from available foods offer the possibility of conducting a proper field study to measure the effectivness of dietary iron supplementation.

At present fourteen grants deal with mineral research amounting to 637 thousand in direct costs or 9.5% of the budget. This area should be expanded to approximately two and one-half this level or a total of 2.5 million by FY 79.

budget. This includes a program project at Johns Hopkins University dealing with the use of keto acids and their salts in treating various metabolic conditions involving nitrogen metabolism or renal failures. These compounds appear to offer the most effective treatment to date for congential hyperammonemia due to urea cycle enzymopathies.

Studies to determine the dietary essential amino acid requirements of preschool age children have completed work on lysine, methionine and tryptophane and will be measuring the needs for isoleucine, valine and threonine.

5. Other Areas

Other areas which have received continued support include the following:

(a) Fat-Soluble Vitamins - 18 grants amounting to 13% of the program budget, including a program project and 7 other grants dealing with vitamin D. The earlier finding that 1,25-dihydroxy form of vitamin $D_3$ was the active form in regulating calcium metabolism has stimulated other studies to test its clinical significance in osteoporosis and related disorders.

(b) Water-Soluble Vitamins - account for 13% of the program budget or 16 research grants. These are basic studies in general, although work on folacin and pantothenic acid are very likely to have applied significance.

(c) Lipids - this area includes 7 grants or 4.8% of the budget, including basic studies on the role of fatty acids and other lipids in metabolism.

(d) Other basic studies - include those on metabolic regulation (8 grants), digestion and absorption (2 grants), nutritional requirements (2 grants), and undernutrition (2 grants). Together, these amount to 14% of the nutrition program budget.

US-Japan Malnutrition Panel

The Malnutrition Panel of the US-Japan Cooperative Medical Science Program included 12 on-going grants and 3 contracts, with the cost to NIAMDD of approximately 500 thousand during FY 77. The studies deal with nutrition in the following priority areas: (1) Host and Environmental Factors - with studies of amino acid requirements in children; of the nutritive value and metabolism of rice and potatoes; and of the prophylactic effectivness of orthophosphates in bladder stone formation; (2) Infection - with studies of the effect of malnutrition in man on secretory mechanisms; on the persistence of measles immunity; and on newborn immunocompetence; (3) Brain Development and Behavior - with basic studies on changes in RNA, RNAse, DNA polymerase and polyamines during various states of nutrition in animals and man; with continuation of research in monkeys ranging from histochemical changes in the developing malnourished brain to behavioral consequences in learning and social interactions; and initiation of studies on the effects of iron deficiency on behavior and performance in man.

During the year, the Panel members made site visits to Bangladesh and to Thailand for the purpose of evaluating their future potentials and strategies for nutrition research. Suggestions were formulated in Dacca for the proposed expansion of the Cholera Research Laboratory to include research on Population and Nutrition. In Bangkok and Chiang Mai, assessments were made of their progress and the country's evolving plans for a National Nutrition Policy and a Consortium for Nutrition Research.

At their annual joint meeting in Sendai, Japan the Panels held a milestone Symposium on Inborn Errors of Metabolism in Southeast Asia. This will serve as a major information base on these problems in that part of the world.

The US Malnutrition Panel is planning to co-sponsor an international Conference on "Behavioral effects of energy and protein deficits" in Washington, DC on November 30 - December 2, 1977, with the Japanese Panel and the Food and Nutrition Board's Committee on Nutrition, Brain Development and Behavior, NRC, National Academy of Science.

Contract Support and Workshops

The NIAMDD has supported the National Academy of Science's Food and Nutrition Board by contract to continue the evaluation of human nutrition requirements. In addition, steps are being taken to support the Board in reviewing the "Dietary Goals for the United States, prepared by the Senate Select Committee on Nutrition and Human Needs" in order to identify major information gaps which deserve priority research attention.

The nutrition program is collaborating with the Fogarty Center in the support of (1) an obesity workshop at NIH, October 20-22, 1977 and (2) travel to the Western Hemisphere Nutrition Congress V, August 15-17, 1977 at Quebec, Canada.

Official Duties

Dr. Gerald F. Combs, Nutrition Program Director was assigned part-time as NIH liason to the Executive Office of Science and Technology Policy to assist in the assessment of the research needs in human nutrition, development of national policy options and identification of specific actions which the Federal Government may take to improve the nutritional health and quality of life of people both in the USA and in less developed countries. Much of this is being done in conjunction with the NIH Nutrition Coordinating Committee.

## Kidney, Urologic and Blood Diseases Program

The research efforts of the Kidney, Urologic and Blood Diseases Program of the National Institute of Arthritis, Metabolism and Digestive Diseases (NIAMDD) are centered on support of fundamental research into basic mechanisms of the relevant biological systems in health and disease. This support is primarily through research grants. In order to carry out more effectively this responsibility, evaluations and analyses of research efforts have been undertaken, usually with consultation from experts in the relevant scientific disciplines. The major evaluation, in process of publication, is the Evaluation of Research Needs in Nephrology and Urology. A new evaluation study is planned for the Hematology Program. Another major effort is to keep lines of communication open with scientists in the field as well as with those with common interests in other government agencies.

Stimulated by findings of the Evaluation Study of Research Needs in Nephrology and Urology, three different projects have been started to acquire much-needed data about the incidence and prevalence of kidney and urinary tract diseases. The first was the award of a contract to the Kaiser Foundation to study Renal Disease and Prevalence Factors. The first year report of this study includes a massive amount of data about outpatients and initial analyses of this data. Inpatient studies and multiphasic health examination data are being analyzed currently. It is hoped that a number of scientific papers will be written about the results of this study in the next year or two. The second study being conducted in collaboration with Lt. Col. Richard Hodder, Walter Reed Army Institute of Research, is of the data on members of the three military services which is being obtained from computer tapes, correlated and collated for comparison and analysis. The third project will be done in collaboration with the Division of Analysis of the National Center for Health Statistics (NCHS) and with the assistance of an IPA physician who has training in public health. Additionally, under an interagency agreement with NCHS, funds have been allotted to support some of the laboratory tests necessary for the kidney and urinary tract aspects of the current NCHS Health and Nutrition Survey.

To continue stimulation of new and ongoing research concepts by support of small conferences in areas of special relevance, this year three conferences received program support: The First International Symposium on Biology and Chemistry of Basement Membranes; The Third Workshop on Vitamin D; and, the Third International Conference on Proteins of Iron Transport and Storage. Plans are underway for a meeting in 1978 about Immune Mechanisms of Kidney Disease, which has especial relevance to problems of glomerulonephritis and other nephritides responsible for a majority of cases of end stage renal disease.

The Evaluation Study of Research Needs in Nephrology and Urology, conducted under a contract with the University of North Carolina principal investigators, Drs. Carl W. Gottschalk and William E. Lassiter, is in the final editing and printing phase and should be completed by the fall of 1977.

The monograph from the meeting sponsored by the NIAMDD on Prevention of Kidney and Urinary Tract Disease in May 1976 will be ready for publication as soon

the almost completed summary recommendations have been collated by the editorial committee. A Data Book on Research Support of Kidney and Urinary Tract Disease in FY 1976 has been produced. This includes not only an analysis of NIH support of kidney and urinary tract disease research support, but also an analysis of that supported by the Veterans Administration and by the military Research and Development Commands. It also has data about the number of patients being treated for end stage renal disease with Federal funds and future projections made by the Social Security Administration.

The program has presented information about its research support to meetings of a number of professional organizations: the Executive Committee of the American Society of Hematology; a joint meeting of the American Society of Nephrology and the National Kidney Foundation; the American Association of Clinical Urologists, the Research Committee of the American Urological Society, Coordinating Council of Urology; and the newly formed Intersociety Committee of the Nephrologists and Urologists.

Cooley's anemia, which in the United States is found predominantly among individuals of Mediterranean origin, was given special Congressional attention through the Cooley's Anemia Act of 1972. This Act has been superseded by recent Genetics Legislation (Title IV of PL 94-278). However, the Inter-Agency Coordinating Committee which was formed in 1972 as part of the implementation of the Cooley's Anemia Act produced its Fourth Annual Report to Congress on the administration of the related Public Health Service programs for FY 1976. Further, an informational meeting was held in November, 1976, to facilitate exchange of information with interested citizen groups about responsibilities and work related to Cooley's anemia conducted by the various government agencies. The first three years' progress of contractors developing and screening iron chelators for the treatment of Cooley's anemia have been evaluated by an external panel of experts, and recommendations for the continuation phase made. A Request for Contract Proposals based on those recommendations was issued, and new contracts will be awarded in FY 1977.

Plans are being drawn up and recommendations made for an Evaluation of the NIAMDD Hematology Program, its ongoing research and future directions for research supported.

Cooperative efforts with other institutes of the NIH which share similar research interests have been continued. Formally, the program participates in the NIH Coordinating Committee for Blood-related Research. Informally, there is cooperation with other institutes who have interests in kidney and urinary tract research, especially those in NHLBI and in NIAID.

## 06 -- Hematology Program

The Hematology Program of the National Institute of Arthritis, Metabolism and Digestive Diseases supports extramural investigation into hematologic disorders ranging from studies of basic mechanisms of normal function and disease pathogenesis through development of treatment modalities to clinical application and evaluation of treatment.

Hematologic disorders which are of major importance to the program are:

1. genetic anemias, particularly the hemoglobinopathies, related to regulation of hemoglobin and hemoglobin synthesis (e.g., Cooley's anemia, sickle cell anemia).

2. metabolic disorders related to iron transport and storage (hemosiderosis), prophyrin metabolism (porphyrias), and enzymatic defects (red cell glucose-6-phosphate dehydrogenase deficiency).

3. nutritional anemias, especially those involving iron, folic acid, and vitamin $B_{12}$ deficiencies.

4. disorders of blood cell production, such as those encountered in systemic and/or metabolic disorders. Examples are the anemia of chronic renal failure, aplastic anemia, and polycythemia.

5. immunology of hematopoietic tissue transplantation and of autoimmune hematologic disease.

Studies on these disorders add to the pool of knowledge related to blood. Additionally, the availability of blood facilitates study of membrane phenomena, protein structure and function, etc., which is broadly applicable across the spectrum of medical problems, such as chronic renal insufficiency, transplantation, and malignancy.

The Hematology Program budget for support of extramural research and training represents about 7.5 per cent of the total NIAMDD extramural budget for FY 1976.

### Genetic Anemias.

Cooley's anemia (thalassemia) and sickle cell anemia are the primary thrust of 48 research projects supported by this program. They include research on genetic regulation of hemoglobin synthesis, hemoglobin structure/function relationships, form, function and demise of the sickled or otherwise defective red blood cell, prenatal diagnosis of conditions on the basis of hemoglobin or other metabolic analysis, and metabolic consequences of the anemias.

In order to understand hemoglobin disorders so that rational bases for prevention can be obtained, it is necessary to develop information about the

structure, function, and genetic regulation of the synthesis of hemoglobin in a variety of its forms. For example, comparative amino acid sequence studies of hemoglobins from patients with various hematologic disorders give useful information about structure/function relationships. Several laboratories which are receiving support for this work, in addition to performing basic research, have developed: 1) the sophisticated technology required, 2) continue to train other investigators and technical personnel in the complex techniques involved, and 3) act as a clearinghouse for the identification and structural analysis of new hemoglobins.

Correlation of structure (e.g., amino acid composition) and function (tetramer formation and oxygen transport) is a complex endeavor which grantees are investigating. Using cysteine residues as sites for probes (spin-labels) which are sensitive to changes in protein conformation, one investigator is able to obtain information on the importance of these specific residues as well as monitor changes which may take place within the molecule during function and characterization. Cysteine residues are used because their presence does not vary in normal and in abnormal human hemoglobins.

One of the most common hemoglobinopathies is sickle cell disease, which results from the predominance of a defective form of hemoglobin (HbS). HbS is relatively unstable, tending to aggregate under lowered oxygen conditions. Aggregation deforms the red blood cells, producing bizarre "sickled" shapes which impede blood flow and interfere with gas exchange in the tissues. Much basic research focusses on the structure of HbS, on genetic and other mechanisms of regulation of hemoglobin synthesis, on factors related to HbS aggregation, and on red cell deformability.

One study has shown that the lowered oxygen affinity of homozygous HbS cells and osmotically shrunken heterozygous cells is directly correlated with their sickling propensities upon partial deoxygenation. This supports the concept that the low oxygen affinity is a direct consequence of HbS polymerization.

The development of therapeutically effective anti-sickling agents for sickle cell disease is the goal of many laboratories. The intermolecular association of deoxy-HbS molecules theoretically could be interrupted by the administration of agents which render the deoxy-HbS more soluble and less likely to aggregate. Using X-ray techniques, investigators have shown that HbS crystals are composed of double strands of molecular hemoglobin with a side-to-side contact involving one of the two valine residues in a known region. This contact site is intriguing because it involves the mutation site in HbS, and offers a possible relationship to exploit the design of anti-sickling agents.

Since the basic mechanism underlying hemoglobinopathies such as sickle cell anemia and thalassemia is an abnormal hemoglobin synthesis, an effective treatment or cure could result from the modulation of genetic control over the synthetic process. Interest has heightened in the possibility of induced augmentation of net synthesis of fetal hemoglobin (HbF), as a possible therapeutic measure. There are circumstances where HbF persists in adult humans, and confers a survival advantage on the red cells, possibly through a dilutant effect on the abnormal adult hemoglobin. The fetal-adult switch in hemoglobin proportions has been examined in animal model systems including hamster, mouse,

frog, sheep and goat, and addition of agents such as erythropoietin has resulted in correlated amounts of HbF synthesized. However, a major effort will be required before enough information is gathered to provide a rational basis for therapeutic techniques.

Research on Cooley's anemia has proceeded along two general themes -- improvement in therapy, and elucidation of basic mechanisms in order to develop preventive measures. The thalassemic disorders result from an imbalanced hemoglobin alpha and beta chain synthesis; therefore, much research has focussed on the identification of a specific abnormality in the pathway of hemoglobin synthesis. The conclusion has been reached that globin translation is normal, the defect probably occurring at an earlier step such as DNA transcription. Analysis of hemoglobins produced in various thalassemic disorders and in normal individuals has provided evidence that normal globin chain synthesis is regulated in a manner to promote equal rates of synthesis of the alpha and beta chains, with the alpha chains representing a major mediator of the beta chain biosynthetic control. This control is ineffective in thalassemia.

Progress has been made in determining the nucleotide sequence of human globin messenger RNA (mRNA) as a result of a new methodology which allows direct sequencing of complementary DNA (cDNA) obtained by copying the mRNA sequence into DNA using viral reverse transcriptase. This type of sequence information should provide a basis for future studies on control or modification of the biochemical and clinical manifestations of thalassemia.

Based on previous and continuing experimental programs, prenatal diagnosis of thalassemia and of sickle cell anemia has been shown to be practical in the hands of skilled practioners. One investigator reports 42 cases of prenatal diagnosis with successful identification of 6 cases of homozygous beta thalassemia and 2 of sickle cell anemia. Pregnant mothers at risk who might have chosen to terminate the pregnancies have continued them.

The Hematology Program has continued to sponsor development of drugs for therapeutic use in patients with chronic iron overload due to repeated transfusion therapy for Cooley's anemia. The effort has consisted of the award of five contracts, two of the contractors responsible for synthesis of new iron chelators based on chemical and pharmacological principles and for characterization of these agents with physical-chemical procedures. One contractor has concentrated on multidentate ligands derived primarily from the phenols, carboxylic acids and tropolones, the other concentrating on hydroxamic acid derivatives. A third contractor has isolated iron-binding compounds from bacterial sources. These and other submitted compounds have been screened in a transfused mouse assay developed by a fourth contractor. Screening is based on ability of the compounds to promote iron depletion in liver and spleen, iron excretion, and general toxicity. Nearly 100 compounds have been screened since initiation of the program in 1974. Several have appeared promising enough to evaluate for animal toxicity, and these studies are planned. A fifth contract has sponsored a limited clinical trial of the chelating agent 2, 3-dihydroxy benzoic acid, which may have potential for clinical use, possibly in combination with other chelating agents.

Metabolic Disorders.

Porphyrin metabolism bears a relationship to hemoglobin, iron metabolism, and to the disease entities referred to as porphyrias. Some work is aimed at elucidating the regulation of heme and hemoglobin synthesis, since little is known of the structure, function and mechanisms of the enzymes involved. Porphobilinogen synthase is an 8-subunit protein which may prove useful in general studies of protein structure including subunit-subunit interaction, kinetics of assembly of subunits, and the nature of the active site. Under grant support, the enzyme has been purified and immobilized by linking to Sepharose. The immobilized enzyme is rendered more easily studied, and is capable of producing hundreds of grams of an intermediate in heme synthesis which is difficult to synthesize and to obtain from natural sources, mainly the urine of patients with acute porphyria.

Several types of hereditary porphyria are known in humans, and are under study in research projects sponsored by this program. A dermatological disorder, erythropoietic protoporphyria (EPP), leads in later life to liver failure due to production of excess protoporphyrin (PP). Grant-supported investigation has indicated that the bulk of the excess PP in these patients is derived from the red blood cells. Preliminary clinical studies show that it may be possible to prevent the eventual liver damage by suppressing release of PP from the red cells, using various drugs.

Recent discovery of hereditary protoporphyria in cattle, the first non-human model of any type of hereditary porphyria except EPP, has implications for biochemical and therapeutic studies. These animals are being investigated extensively to characterize their disease and to provide background information for experimentation.

Support for studies on iron metabolism, transport, and storage represents a sizeable portion of this program, amounting to nearly $1 million in FY 1976. Emphasized are studies on the mechanisms of iron binding to carrier proteins, subsequent distribution of bound iron, and causes and mechanisms of pathologic conditions of iron transport and storage. This support is directed towards the development of means for prevention and treatment of abnormal conditions.

These studies have established that hemoglobin and other heme compounds absorbed from intestinal lumen are split in the mucosa by heme oxygenase, with a resultant release of heme iron. Iron deficiency in rats results in increased mucosal heme oxygenase activity, and this in turn is associated with enhanced heme iron absorption. The released iron probably is incorporated into the mucosal iron pool and then is transferred to the portal blood. This evidence substantiates the hypothesis that the ability of the intestine to absorb and to transport iron is determined not by body iron stores, but by the amount of nutritionally available iron to which the intestinal mucosa has become conditioned. Absorbed iron is bound to a number of protein types under study, including transferrin, ferritin, and hemopexin. Ferritin is particularly abundant in liver, spleen, bone marrow and other tissues involved in hemopoiesis or in red cell breadown. In addition to acting as a safe, temporary store for iron, ferritin may have regulatory functions in view of its enzymatic properties in the oxidation and deposition of iron and the puzzling presence in some

tissues of ferritin molecules that contain little iron. Multiple forms of ferritin that differ structurally and metabolically (isoferritins) have been found in many mammalian tissues. Their function/structure relationships are under investigation. The isoferritin profiles in human tissues have been shown to be markedly altered in cancer, a finding of potential diagnostic use. For example, certain isoferritins of a more acidic nature than those in the normal adult liver have been found in hepatocellular carcinoma. Similar acidic isoferritins have been described in other carcinomas. More recently, evidence indicates these isoferritins may not be specific for cancers, but may correspond to the more acid isoferritins commonly found in normal adult heart.

There is a need to develop a rational basis for the use of the newly developed serum ferritin immunoradiometric assay as a valid indicator of iron stores in the human. Good correlations between serum ferritin and liver ferritin may be observed only when liver damage has been provoked by environmental stimuli (such as injection of iron). Ordinarily, however, a relatively poor correlation is observed within the normal range, and does not allow serum ferritin measurements to be used with the same degree of confidence as tissue ferritin determinations. Attempts to develop a rational basis for this assay are in progress. It is likely that much more knowledge of a fundamental character must be obtained before such assays might be meaningfully applied to clinical situations in man.

Numerous clinical examples of iron overload conditions, resulting in pathologic iron stores, are known. The mechanisms underlying these conditions are under investigation in several projects. One study has shown enhanced binding of transferrin to red cells containing iron deposits (sideroblastic) as an aspect of the mechanism. Characterization of the receptor site has demonstrated the significance of the carbohydrate protion of membrane polypeptides.

Nutritional Anemias.

As obtained in the Health and Nutrition Examination Survey, US, 1971-1972 (DHEW/HRA), the U.S. population at all age levels exhibits a deficiency of the nutrient iron, based on both dietary intake and biochemical data. The biochemical iron deficiency has a higher prevalence among children, but iron deficiency anemia is widespread among older persons as well. Assessment of iron stores by measurement of transferrin is a useful indicator of anemia in older persons. However, the assay is not satisfactory in the case of young children, whose iron stores are not yet developed. Research is being sponsored to develop a sensitive predictor of iron deficiency anemia in children, in order that iron therapy can be administered before anemia develops. A likely predictive test is based on correlation of free erythrocyte porphyrins (FEP) and hemoglobin (Hb), FEP elevation occurring just before the development of anemia.

In an effort to improve iron nutrition, various iron supplements have been investigated. Phosphoproteins, such as those found in egg yolk and milk, have been implicated as causal agents in the poor absorption of iron. One study has shown that at least with some of the phosphoproteins, no chemical or nutritional basis for this implication can be found. The investigator also has found superior absorption and assimilation of such supplements as ferric fruc-

tose and ferric citrate, as compared to that for standard doses of the common iron supplement ferrous sulfate, with none of the latter's toxic side effects.

Research on folic acid and vitamin $B_{12}$ metabolism is also of importance in development of an understanding of nutritional anemias. Sponsored research has suggested that doses above the Recommended Dietary Allowance of 45 mg of ascorbic acid (vitamin C) destroy substantial amounts of vitamin $B_{12}$ in food and bile, with a significant possibility of vitamin $B_{12}$ deficiency disease developing. This is of significance in view of reports that over one million Americans take large doses of vitamin C as a home prophylaxis and remedy for the common cold.

Vitamin $B_{12}$ and folic acid interact in red blood cell production during methionine synthesis, with folate acting as a single carbon group carrier. A well-recognized gap in knowledge is the extent to which the human population suffers from folate deficiency. The role played by an inadequate supply of dietary folate and the physiological significance of folate depletion is not known. Research supported by this program is aimed at the development of model systems such as lymphocyte cultures as tools for determination of the coenzyme function of folate in the human population.

Disorders of Blood Cell Production and Destruction.

Investigation into mechanisms of production, destruction and function of blood cells is supported . The physiological regulator of red cell production, erythropoietin (Epo), acts on marrow cells to produce an active cytoplasmic factor that stimulates RNA and subsequent DNA synthesis.

Research in erythropoiesis has been hampered by the lack of methods for purification of Epo. Since only incompletely purified preparations of Epo are available, the precise structure of the hormone is unknown, and even the most highly purified preparations carry contaminants which can lead to difficulties in interpretation. A significant advance has been made by one investigator, who has developed a technique of affinity chromatography with agarose-bound lectins to isolate Epo, and has obtained much better purification. This technique is being refined in this investigator's laboratory as well as in many others.

The role of the kidney in producing Epo in response to a reduced red cell mass is well known, but the mechanism of production is not clear. In conditions such as chronic renal failure where Epo production is diminished, it may be possible to prevent the accompanying anemia by stimulating Epo synthesis with hormonal or other drug treatment. Studies of the effectiveness of administering exogenous Epo to renal patients with anemia are being supported.

Bone marrow, the main target of Epo, is under extensive study. The marrow contains hematopoietic tissue separated by large thin-walled sinuses which deliver blood cells to the circulation. The Epo-induced movement of cells through the walls of these sinuses is significantly impaired in hypertransfusion, possibly due to increased intrasinus pressure in the lumen which is packed with erythrocytes. This activity and others related to bone marrow function, such as

mechanisms of drug-induced bone marrow aplasia and the mobilization of white blood cells in marrow are under investigation.

Immunology and Transplantation.

With the increase of patients receiving organ and tissue transplants, great interest has arisen in immune reactions associated with white cell surface antigens. One project is designed to study the genetics, immunology and cellular basis of the hempoietic-histocompatability system (antigens selectively expressed on hematopoietic cells), immune responsiveness to such antigens, and their role in resistance to bone marrow transplantation. The long-term goal of this study is to develop assays to type and match prospective donors of marrow. Considerable progress has been made in understanding the genetic basis for hemopoietic incompatibility in mice, and in developing an *in vitro* model system for graft rejection.

White cell antigens also have been associated with resistance to a broad spectrum of diseases and infections, including juvenile rheumatoid arthritis, ankylosing spondylitis, multiple sclerosis, inflammatory bowel disease, periodontitis, etc., and these diseases may be correlated with a defective immune response (IR). Establishment of IR genes, such as those for human lymphocyte antigens (HLA), in relation to particular diseases, may help to assess an individual's potential susceptibility, and thus provide an aid for prevention.

Other projects deal with complement synthesis, structure, and role in hemolysis, the immunologic nature of immune thrombocytopenia, and development of a sensitive solid phase type of radioimmunoassay for characterization of glycoproteins from erythrocyte and thymocyte membranes.

Training.

Only four institutional training grants were active in FY 1976 as these continue to phase out. Eight Institutional National Research Service Awards were active, with slots for 27 postdoctoral trainees. Eight individual fellowships were active.

## 12 -- Kidney Disease and Urology Program

The Kidney Disease and Urology Program encompasses research relating to the kidney and urinary tract. The Program's activities are concerned with basic aspects of the etiology, physiology, pathophysiology and biochemistry of relevant disease entities. These efforts include studies of the cause, prevention, diagnosis and treatment of renal and urinary tract disorders which include nephritis, nephrosis, benign prostatic hyperplasia, polycystic disease, diabetic nephropathy, urolithiasis, glomerulonephritis, pyelonephritis and other nephritides. The Program also fosters support of basic and clinical research relating to dialysis, renal transplantation (including organ preservation and storage); and of renal function, including metabolic and transport mechanisms and disorders.

There are overlapping areas of interest with other institutes with the NIH in the mutual concerns about end-stage renal disease, transplantation, pyelonephritis, glomerulonephritis; electrolyte balance, vascular components of kidney structure and function; the paraplegic bladder among others. There also exists overlap with other programs within the NIAMDD relating to studies about systemic lupus erythematosus, diabetic nephropathy; chronic renal failure, dialysis; anemia of uremia, and calcium and phosphorus metabolism. Research support of these efforts in the form of research grants, program project and Research Career Development Awards rose to $16.407 million in FY 1977. An additional $1.5 million was made available to initiate a program of Specialized Centers of Research (SCORs) in Urolithiasis, in which it is anticipated that four SCORs will be funded in FY 1977.

Basement Membrane Diseases.

The physiology and metabolism of renal basement constituents or constituents from renal tubular cells are areas about which little is known. Furthermore, antibasement membrane antibody induced diseases, including glomerulonephritis and Goodpasture's Syndrome are being diagnosed with increasing frequency. One group at the Scripps Clinic and Research Foundation currently is evaluating 300 such patients in another study in which the disease is frequently fatal or results in irreversible renal failure requiring dialysis or transplantation. Little information is available about the sequence of events which trigger the spontaneous formation of antibasement membrane antibodies in man. The studies outlined for a three year period are designed to study the pathophysiology of the nephritogenic basement membrane antigens in animals. These studies are aimed to establish events that might alter the normal processing of these basement membrane antigens which result in the establishment of anti-basement membrane antibody immune responses. With an understanding of the events that trigger the anti-basement membrane immune responses, it is anticipated that progress can be made toward prevention or modification of these diseases.

Protein Handling by the Kidney.

In health, the kidney effectively conserves albumin and other plasma proteins. In renal diseases such as glomerulonephritis, proteins appear in the urine in abnormal amounts. The precise way in which protein conservation occurs in health and protein loss in disease is poorly understood. In the past few years new developments in the concepts of the relationship of glomerular permeability to the net charge of molecules has prompted the view that albumin loss in the disease state is caused by changes in the polyanionic nature of the glomerular basement membrane. Due to advances in protein chemistry during the past ten years, it has become possible to test this hypothesis by modifying the net charge of proteins. An investigator at the University of Cincinnati is modifying human albumin by adding ethylene diamine to the carboxyl moieties of glutamyl and asparate protein residues. The altered protein has been prepared in gram amounts and characterized by physiochemical and immunologic procedures. These studies with modified albumin derivatives are continuing and should establish whether or not protein charge influences glomerular permeability and protein uptake.

Nephrosis.

Progress in the understanding of the experimental kidney disease known as puromycin aminonucleoside induced nephrosis (PAN) continues. The most dramatic ultrastructural alteration associated with PAN is the loss of pedicels (foot processes) from kidney podocytes. This change is believed to involve the retraction of pedicels and replacement of interpedicular slit pores with occluding junctions. For over a decade investigators have differed about whether or not this loss of pedicels represents an attempt by the visceral epithelial cells to "monitor" excess protein which is escaping across the glomerular basal lamina. A grantee at the University of Texas Southwestern Medical School postulates that he has resolved this dilemma by studying changes in the visceral epithelium during the onset and progression of aminonucleoside induced proteinuria and comparing these changes with those which occur during a similar degree of proteinuria induced by injections of albumin. The results of his investigation indicate that loss of podocyte pedicels during PAN does not appear to be a secondary response to the proteinuria sssociated with the disease. These findings suggest that the process of pedicel loss may be more intimately associated with the cause of PAN proteinuria than had originally been speculated.

Acute Renal Failure.

Research on Acute Renal Failure (ARF) induced by toxic agents of injury has elucidated mechanisms which could have a direct bearing on means to prevent environmentally-induced kidney damage. Models of toxic injury produced by such substances as uranium, serine, uranyl nitrate, and dichromate have been developed by grantees to study the nature of toxic injury. Depending upon the dose administered, a characteristic tubular necrosis with oliguria can be produced, which is followed by repair with polyuria and finally, complete recovery of structure and function of the nephron. Work in a laboratory at

the Kansas University Medical Center has established the morphology of the regenerative porcess as stemming from residual cells within the necrotic zone by rapid replication and later differentiation. Nevertheless, in most forms, there remains uncertainty as to the precise mechanism of the cell injury, the manner in which the toxic substance is incorporated into the tubular epithelium, and the means of production of the oliguria.

Most present concepts of the generation of ARF require the presence of renal ischemia as a major contributing factor during the initiating stage. A number of investigators have demonstrated that renal ischemia is present in the initial stage of uranyl nitrate induced acute renal failure. However, a group in San Antonio has demonstrated that uranyl nitrate did cause acute renal failure without the concomitant renal ischemia in dogs. This suggests that the drug has a direct effect on tubular function. In rat experiments involving dichromate, the data suggests that the recovery process occurs in two stages. In the first stage there is restoration of outer cortical perfusion and glomerular filtration, whereas in the second stage there is restoration of total renal blood flow and tubular function. It is anticipated that these studies will provide a better understanding of the mechanisms involved in the recovery process following acute renal injury and lead to improved therapeutic measures.

Viral Infections.

Cytomegalovirus (CMV) infections are known to pose a threat not only for renal transplant patients but for bone marrow transplants and patients with lymphoproliferative malignancies. Prospective studies at the University of Minnesota have suggested that the finding of leukopenia is useful for the early recognition of CMV infections in renal transplant patients. In particular, this group's prospective virologic surveillance has shown that the onset of CMV infection is usually in the second post-transplant month and is often heralded by leukopenia. Thus, early recognition of CMV infection may be very helpful to the transplant surgeon in the management of the patient's immunosuppression regimen. This group also suggests that the association of leukopenia with CMV syndrome may be valid for bone marrow transplants and leukemia patients who frequently develop serious CMV infections.

The Minnesota group contends that if differences among CMV strains can be detected by serologic techniques, it will be possible to trace a single virus from patient to patient, from donor to renal allograft recipient, or from patient to hospital personnel. Since a majority of their transplant patients develop CMV infection within six months post-transplant, they became particularly interested in the epidemiology of CMV. Therefore, in October 1976, a screening program was begun on 62 members of the transplantation service at the University of Minnesota and it is anticipated that the continuation of this surveillance study over the next four years will provide answers to very important epidemiologic questions.

Urinary Tract Stones.

Infection-induced urinary tract stones are a major urological problem. Urinary tract stones that form as a consequence of urinary infection account for approximately twenty percent of all urinary tract stones. In addition, the morbidity and mortality that result from infection-induced stones are considerably higher than all other forms of stones. Clinical reports over the past 50 years have implicated urea-splitting bacteria as the primary causative agent for increasing urine alkalinity and ammonia concentration which in turn cause supersaturation with both struvite and calcium phosphate.

A known effective inhibitor of the bacterial enzyme urease, acetohydroxamic acid (AHA), is being actively studied in two laboratories in Houston, one at Baylor College of Medicine and the other at the University of Houston. Investigators at the University of Houston are examining the pharmacokinetics and bioavailability aspects of AHA in man; and the absorption, distribution and elimination kinetics of AHA in rats to determine the approximate dosage regimen for treatment therapy of patients with infection stones.

The Baylor group is engaged in a long term clinical study, involving treatment of patients with AHA, in an attempt to dissolve urinary tract stones in patients who chronically form them. In another group of patients, who have been rendered surgically stone free, but who still harbor chronic, recalcitrant urea-splitting urinary infection, AHA is being administered in an attempt to evaluate its effect on stone recurrence. These investigators have shown that a group of 23 patients who have taken AHA for periods of 6-18 months can tolerate doses of 1 gram per day and that the urinary ammonia and pH are reduced in every patient receiving the drug. Stone growth and/or stone recurrence have not been observed in any patient receiving AHA. Although hemolytic anemia was a side effect of the drug in three patients, the anemia was found to be dose related and was completely reversible on lowering the dose of AHA. These three patients are continuing in the study but are being treated with smaller doses of AHA.

Prostatitis.

An investigator at the University of Wisconsin has received a grant to support studies of the concentration of various antibacterial agents in the chronically infected prostate. Antimicrobial substances that are known to concentrate in prostatic fluid and tissue will be given preoperatively to patients undergoing prostatectomy for benign prostatic hyperplasia, with and without concomitant chronic prostate infection. The tissue removed at surgery will be analyzed. The results of these studies should be helpful in providing guidelines for therapy and/or prevention of bacterial prostatitis.

Cystitis.

A grantee at the University of California, San Francisco, has been engaged in studies which are directed at the eventual control of recurrent cystitis over the past 10 years. The investigator is attempting to delineate the factors

which promote selective bacterial adherence and local colonization in the lower urinary tract. Studies are being made of means to improve surface defenses of the urothelium that could prevent the invasion and adherence of cystitis-causing bacteria.

Drug Trials and Clinical Surveys.

Adhering to major objectives of an international multicenter study that was initiated seven years ago by a group at Albert Einstein, this group is continuing to conduct clinical surveys and randomized therapeutic trials on the major kidney diseases of children. The results of these trials and surveys are providing, in many instances for the first time, a scientific basis for treating and describing the course of most of the major renal diseases in children. Thus far, data has been accumulated on natural history and treatment of glomerular diseases in children, natural history and the adrenocortical glucosteroid treatment of the nephrotic syndrome in children and histopathologic criteria for assessing change in membrane proliferative glomerulonephritis.

Kidney Preservation.

At the University of Wisconsin a group of investigators have identified at least two defects in the perfused kidney that are potentially harmful to prolonged organ preservation. These include imbalance in the distribution of cellular water and a loss of total adenine nucleotides (TAN). To alter the level of TAN in the perfused kidney, these investigators added a number of compounds that are involved in the synthesis and breakdown of these high energy compounds, and were able to demonstrate that they are partially effective in altering TAN levels in only some instances. The results, are inconclusive as far as recommendations at the present time for successful organ preservation are concerned.

Training.

An additional six new institutional National Research Service Awards (T32s) were funded in FY 1977, bringing the total to 24 active training programs which provide stipends for 64 postdoctoral trainees. In addition, 21 individual fellowships (both F22s and F32s) were active during this period and one new Research Career Development Award was awarded, maintaining the number at 9.

Specialized Centers of Research in Urolithiasis.

In response to one of the major recommendations outlined in the Evaluation Study of Research Needs in Nephrology and Urology and supported by the deliberations of several recent conferences on urinary tract stones, an effort has been made to markedly increase research efforts in urolithiasis by making $1.5 million available in FY 1977 to support 3-4 Specialized Centers of

Research (SCORs) in Urolithiasis. Since the etiology in the majority of patients with urolithiasis is not understood, it is anticipated that by delineating basic mechanisms responsible for this malady, the possibility exists that this intensified effort may ultimately lead to a means to prevent or to arrest the course of stone formation. The major research approaches of these SCORs are being directed at metabolic, biochemical, and physiological aspects of calcium, phosphate, oxalate, and cystine transport, vitamin D metabolism and parathyroid physiology. The emphasis of these SCORs is upon fundamental research.

First International Symposium on the Biology and Chemistry of Basement Membranes.

The National Institute of Arthritis, Metabolism and Digestive Diseases provided partial support of the Symposium on the Biology and Chemistry of Basement Membranes, which was convened on November 29-December 1, 1976 at the University of Pennsylvania, Philadelphia. This timely meeting attracted some 100 experts from the U.S. and abroad to exchange ideas on the new concepts of the role of basement membranes in a number of disease states, including glomerulonephritis, Goodpasture's Syndrome, scleroderma, rheumatoid arthritis, and diabetes mellitus. The Symposium afforded the opportunity to review the research in the field and establish new approaches for future investigations.

OFFICE OF THE ASSOCIATE DIRECTOR FOR PROGRAM ANALYSIS
AND SCIENTIFIC COMMUNICATION

SCIENTIFIC COMMUNICATIONS SECTION

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Workshop on Collagen Metabolism in the Liver

Workshop on Vitiligo

The Scientific Communications Section has responsibility for general programming and planning of communication activities concerned with various scientific areas charged to NIAMDD. The present activities can be generally grouped into four main categories.

1. Current awareness literature services;
2. Retrospective literature studies;
3. Conferences and workshops; and
4. Other aspects of communication, such as the production of motion pictures, thesaurus development, etc.

Current awareness literature services are provided by the Institute in several specialized areas. At present, there are four publications with hierarchically arranged listings of literature citations and one publication which includes abstracts. Gastroenterology Abstracts and Citations, now in its tenth volume, is intended to provide selected abstracts and literature citations in all aspects of gastroenterology, biochemical or clinical. Four of the publications, Kidney Disease and Nephrology Index (third volume), Endocrinology Index (tenth volume), Diabetes Literature Index (twelfth volume), and Index of Dermatology (ninth volume) contain citations to current world-wide literature which have been included by the National Library of Medicine in their Medical Literature Analysis and Retrieval System (MEDLARS).

To produce structured, hierarchically-arranged indexes which function as in-depth current-awareness tools for easy review of particular subjects, we have cooperated with the National Library of Medicine, and in one case also with the University of Minnesota and the University of Rochester. The standardized terminology used in MEDLARS makes these publications invaluable for retrospective literature searching and to those who are interested in these highly specific areas.

Outstanding scientists working in the field covered by the publications have been assembled for each publication as editorial advisors to insure the continuation of the high quality of these publications.

Kidney Disease and Nephrology Index Volumes I (1975) and II (1976) issues were combined into two large issues for each volume. However, with Volume III (1976), the publication will appear with bimonthly issues.

Conferences, symposia, and workshops sponsored by NIAMDD are planned and organized by the Scientific Communications Section. In addition to the organization of the conference with the conference chairman, (including physical arrangement as well as program planning), the publication of the proceedings also falls under the auspices of the Scientific Communications Section.

This year eleven conferences, symposia, or workshops, sponsored completely or jointly, were held. Conferences and a symposium included the Tenth Annual Contractors' Conference of the Artificial Kidney-Chronic Uremia Program, Conference on Acute Fulminant Hepatic Failure, Conference on Growth Retardation in Children with Kidney Disease, Conference on the Role of Dietary Fiber in Health, Conference on Vitiligo, the First International Conference on Nutriti

in Renal Failure, and the Symposium on Drug Action and Metabolism in Renal Failure. Workshops included the Workshop on Biliary Atresia, Workshop on Blood Access for Maintenance Dialysis, Workshop on Collagen Metabolism in Liver, and Workshop on Sexual Dysfunction in Chronic Renal Disease. Less extensive support, usually along with other Institutes was contributed to conferences concerning vitamin D, training of gastroenterologists in cancer, biology and chemistry of basement membranes, and two international conferences on endocrinology and transplantation.

The Cystic Fibrosis Foundation, with the Institute, has compiled a comprehensive bibliography with indices containing citations to all known literature on various aspects of cystic fibrosis. This definitive Bibliography covering the literature from 1813 to 1972 is distributed through the Cystic Foundation to all with interests in this disease.

The Proceedings-WHO Regional Symposium on Vesicle Calculus Disease was published through the Government Printing Office. This first international conference on urinary bladder stone disease was held in 1972 in Bangkok, Thailand. The Proceedings gave an historical outline of this disease, reports of research findings, and summaries of areas needing research such as basic, clinical, and epidemiological studies.

The Evaluation of Hemodialyzers and Dialysis Membranes is an update of two earlier publications, and provides the latest technology and research advances in hemodialyzers. This publication serves as a reference providing guidelines for researchers involved in the development of dialyzers and clinicians using hemodialyzers.

National Library of Medicine's on-line MEDLARS (Medical Literature Analysis and Retrieval System) is available to NIAMDD staff through NIAMDD's call number. The Scientific Communications Section is responsible for coordinating this use and serves as a reference source for all of NIAMDD staff (Extramural, Intramural, and Administrative).

In addition, numerous other activities that bear on scientist-to-scientist communication as it affects the Institute are dealt with frequently. Areas where further emphasis possibly should be placed are continually in review.

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|---|---|
| Project Title: | DIABETES LITERATURE INDEX |
| Contract Number: | PH43-67-663 |
| Amount: | $101,929 |
| Contractor: | University of Minnesota |
| Project Officer: | Mrs. Billie B. Mackey |
| Man Years: | Total: .40<br>Professional: .20<br>Other: .20 |

Project Description:

A current-awareness publication, the Diabetes Literature Index, produced monthly, is now in its twelfth volume. This index of diabetes-related literature was brought about through the cooperative efforts of the Institute, the National Library of Medicine, and the American Diabetes Association, and three universities, (University of Minnesota, University of Rochester, and Case Western Reserve University), and many individual scientists.

The NLM's MEDLARS data base, containing all of the current biomedical literature citations to both journals and books, is used to retrieve all diabetes-related bibliographic citations monthly. Until 1971, the Diabetes Literature Index, consisting of a keyword in title index and an author index, was prepared from a computer printout. Since 1971, a magnetic tape of these indexes is prepared at the University of Minnesota and is used to generate copy for offset printing by the Government Printing Office on the Linotron. The publication includes three formats, a hierarchical subject index of core diabetes literature, an abbreviated keyword in title index of all diabetes literature, and an author index including index terms with the citations (for the first author). The citations are grouped under a preconceived hierarchical structure with related subjects grouped together making it easier for users to scan their particular interests in one section.

The Scientific Communications Section, NIAMDD publishes the Diabetes Literature Index monthly, and it is available to National Institutes of Health grantees or contractors with interests in diabetes, government agencies with responsibilities in this area, and to medical school libraries. It is for sale by the Superintendent of Documents, Government Printing Office for a fee. The free mailing list was circularized to insure currency.

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|---|---|---|
| Project Title: | ENDOCRINOLOGY INDEX | |
| Professional Personnel: | Mrs. Billie B. Mackey | |
| Man Years: | Total: | .7 |
| | Professional: | .4 |
| | Others: | .3 |

Project Description:

The Endocrinology Index, a bimonthly current-awareness publication, is now in its tenth year. Each issue contains approximately 4,000 citations of the current world literature, both journals and monographs, in endocrinology. This bibliography is produced, with the aid of the National Library of Medicine's Medical Literature Analysis and Retrieval System (MEDLARS), by the Scientific Communications Section, NIAMDD. The format consists of citations grouped in eight categories (pituitary, thyroid, etc.) and each category has a preconceived hierarchical organization of subject headings. Index terms, assigned to the original article by the indexer, appear in the author section, and author and subject indices appear in each issue.

Citations from other sources are selectively added including books and articles of potential interest to neuroendocrinologists chosen from the six publications of the Brain Information Service. Work with the Medical Subject Headings Section of the National Library of Medicine is ongoing for inclusion of endocrine-related vocabulary in the computerized vocabulary.

Endocrinology Index is available to National Institutes of Health grantees or contractors with interests in the field of endocrinology, government agencies with responsibilities in the field, and medical school libraries. It is for sale by the Superintendent of Documents, Government Printing Office for a fee. The free mailing list was circularized to insure currency.

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|---|---|
| Project Title: | GASTROENTEROLOGY ABSTRACTS AND CITATIONS |
| Contract Number: | NO1-AM-4-2204 |
| Amount: | $141,825 |
| Contractor: | Franklin Institute |
| Project Officer: | Mrs. Billie B. Mackey |
| Other Professional Personnel: | Dr. Andrew Greenhill<br>Dr. David Sherman<br>Mrs. Elsie C. Yuen |
| Man Years: | Total: .30<br>Professional: .20<br>Other: .10 |

Project Description:

Since January 1966, NIAMDD has been publishing Gastroenterology Abstracts and Citations, a monthly current-awareness journal for gastroenterologists. Prepared by contract, each issue contains approximately 350 abstracts and 750 citations of the significant world literature on gastroenterology. The abstracts and citations are listed under three main categories: Clinical Sciences, Diagnostic Procedures, and Gastrointestinal Diseases. These categories are subdivided into more specific groupings. Each issue contains subject and author indices which are cumulated annually.

The staff evaluates and edits the abstracts and citations for relevancy and quality prior to publication. As the result of closer monitoring this past year, the editorial format has been upgraded and other improvements made. A meeting of the Editorial Advisors was held this year.

Gastroenterology Abstracts and Citations is available free upon request, to qualified investigators with NIH grants or contracts in the field of gastroenterology, to other government agencies with responsibilities in this area, and to medical school libraries. In addition to its free distribution, this publication is for sale at a nominal price by the Superintendent of Documents, U.S. Government Printing Office. The mailing list was circularized to insure currency.

Project Title; INDEX OF DERMATOLOGY

Professional Personnel: Mrs. Elsie C. Yuen
Mrs. Billie B. Mackey

Man Years:

| | |
|---|---|
| Total: | .5 |
| Professional: | .25 |
| Other: | .25 |

Project Description:

To support the NIAMDD Dermatology Program, and respond to an increased interest and demand for up-to-date clinical studies and research information in dermatology, NIAMDD has been publishing the Index of Dermatology since April 1972.

The Index of Dermatology is a monthly recurring bibliography produced by the Scientific Communications Section with the aid of MEDLARS, the Medical Literature Analysis and Retrieval System of the National Library of Medicine. About 1300 dermatology-relevant citations are computer-selected each month from the Index Medicus input for the corresponding month, and listed under general clinical, biology, and/or cell component sections, and under an author index. The hierarchical format used allows titles in a related subject area to be grouped, on the basis of their clinical or research orientation, under a specific subject hearing.

The staff monitors the retrieval and edits the camera copy of the bibliography prior to publication. To keep the bibliography useful and timely, the staff also draws on the expertise of the Editorial Advisors.

As the result of a meeting with the Editorial Advisors this year, several recommendations were made to the National Library of Medicine to broaden and update the contents of the Index. New retrieval key words from the 1977 MeSH (Medical Subject Headings), the controlled vocabulary authorized by the National Library of Medicine, were added as new subject headings. A new publication Contact Dermatitis was suggested for indexing as were new key words for incorporation in the 1978 MeSH. In keeping with these changes, the staff also updated and revised the search formulation.

The Index of Dermatology is available free to qualified investigators with NIH grants or contracts in the field of dermatology, to other government agencies with responsibilities in the area, and to medical school libraries. In addition to the free distribution, it is for sale by the Superintendent of Documents, Government Printing Office, at nominal cost. The mailing list was circularized to insure currency.

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|---|---|---|
| Project Title: | KIDNEY DISEASE AND NEPHROLOGY INDEX | |
| Professional Personnel: | Mrs. Billie B. Mackey<br>Mrs. Elsie C. Yuen | |
| Man Years: | Total: | .9 |
| | Professional: | .6 |
| | Other: | .3 |

Project Description:

The Kidney Disease and Nephrology Index, is a bimonthly recurring bibliography including biomedical literature citations in the areas of kidney disease, nephrology, urology, artificial kidneys, and kidney transplantation. With the aid of the National Library of Medicine's Medical Literature Analysis and Retrieval System (MEDLARS), this recurring index is produced by the Scientific Communications Section, NIAMDD. Each issue contains approximately 2200 citations arranged hierarchically to group similar subjects together. The format also includes subject and author indices. Other citations of potential interest to nephrologists, urologists, and biomedical engineers are included. A listing of annual and final technical reports submitted to the Artificial Kidney-Chronic Uremia contract program are included. These reports are available through the National Technical Information Service for a fee.

The Kidney Disease and Nephrology Index is available to National Institutes of Health grantees or contractors with interests in nephrology, urology, or artificial kidneys, government agencies with responsibilities in the area, and to medical school libraries. It is for sale by the Superintendent of Documents, Government Printing Office for a fee. The free mailing list was circularized to insure currency.

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|---|---|
| Project Title: | ARTIFICIAL KIDNEY-CHRONIC UREMIA PROGRAM<br>NINTH ANNUAL CONTRACTORS' CONFERENCE PROCEEDINGS |
| Professional Personnel: | Mrs. Billie B. Mackey |
| Man Years: | Total: .4<br>Professional: .2<br>Others: .2 |

Project Description:

The Proceedings of the Ninth Annual Contractors' Conference held in Bethesda, Maryland, January 12-14, 1976 was published. About 250 individuals attended of whom approximately 175 participants were contractors and their staff, consultants to the Artificial Kidney-Chronic Uremia Program, and Institute staff. The sessions consisted of reports of existing contract-funded research and development projects. The presentations were grouped into two simultaneous sessions: (1) biochemistry and pathophysiology and (2) biocompatibility, membranes, and device development. Three panel sessions were held.

The conferees communicated research results in the main areas of the Artificial Kidney-Chronic Uremia Program, and a considerable body of detailed and pertinent scientific and technical information was exchanged between the top staff of the 70 currently active research and development projects, the Program's consultants and its staff.

The Proceedings were given worldwide distribution to those with interests in the fields of nephrology and artificial kidneys. It is available through the National Technical Information Service for a nominal fee.

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|---|---|
| Project Title: | ARTIFICIAL KIDNEY-CHRONIC UREMIA PROGRAM<br>TENTH ANNUAL CONTRACTORS' CONFERENCE AND PROCEEDINGS |
| Professional Personnel: | Dr. Robert J. Wineman<br>Dr. Fernando Villarroel<br>Mrs. Billie B. Mackey<br>Mrs. Elsie C. Yuen |
| Man Years: | Total: .4<br>Professional: .2<br>Other: .2 |

Project Description:

The Tenth Annual Contractors' Conference was held January 17-19, 1977 in Bethesda, Maryland. Approximately 275 interested individuals attended including 170 contractors and their staff, consultants to the Artificial Kidney-Chronic Uremia Program, and Institute staff.

The presentations, consisting of reports of existing contract-funded research and development projects, were grouped in two simultaneous sessions. A session covered carbohydrate and lipid metabolism, acetate in anemia, biochemical pathophysiology, and blood access and biocompatibility. The other session included adsorbents, membranes and devices, peritoneal dialysis, and therapy evaluation.

Research results in the main areas of the Artificial Kidney-Chronic Uremia Program were presented with detailed scientific and technical information communicated among the top staff of the currently active research and development projects. A plenary session included summaries of major points of the most significant findings given during the presentations, and a review of hemofiltration. The Proceedings will be published in the fall.

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| Project Title: | WORKSHOP ON BLOOD ACCESS FOR MAINTENANCE DIALYSIS | |
| Professional Personnel: | Dr. Robert Wineman<br>Dr. David Sherman<br>Mrs. Billie B. Mackey | |
| Man Years: | Total: | .1 |
| | Professional | .05 |
| | Other: | .05 |

Project Description:

Approximately 150 participants attended this workshop held January 16, 1977 in Bethesda, Maryland. An overview of present successes and failures of various blood access techniques for improved dialysis was presented. After a discussion on the extent and mode of access device failure, the attendees considered specific topics pertaining to the properties of materials used in various devices and their interaction with blood, blood flow dynamics in devices and A-V shunts, thrombosis in maintenance dialysis, and vessel wall responses in chronic maintenance dialysis. Comments and discussion attempted to identify the areas of most critical need for future research.

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| Project Title: | WORKSHOP ON SEXUAL DYSFUNCTION IN CHRONIC RENAL DISEASE |
| Professional Personnel: | Dr. Robert Wineman<br>Dr. Andrew H. Greenhill<br>Mrs. Billie B. Mackey |
| Man Years: | Total: .1<br>Professional: .05<br>Other: .05 |

Project Description:

Concern with the quality of life for patients with end-stage renal disease on dialysis led to this workshop held on January 16, 1977 in Bethesda, Maryland. Approximately 150 participants met to discuss the problem of endocrine abnormalities, especially those related to sexual dysfunction in chronic renal disease. Topics covered the incidence of sexual dysfunction in the pre-dialysis, dialysis, and transplant population, differentiating between psychologic and organic etiologies of impotence, pituitary and gonadal hormonal alterations in uremic patients and the value of hormone supplementation, and the correlation between clinical dysfunction and abnormalities. The program concluded that further research was needed.

Project Title: CONFERENCE ON ACUTE FULMINANT HEPATIC FAILURE

Professional Personnel: Dr. Sarah Kalser
Mrs. Billie B. Mackey

Project Description:

Sponsored jointly by the Institute's Liver Diseases Program and the Fogarty International Center, this conference on acute fulminant hepatic failure was held February 7-9, 1977 at Bethesda, Maryland. New findings from several relevant disciplines that affect clinical hepatology were highlighted. The presentations covered fulminant hepatic failure (FHF) with major emphasis: (1) description of FHF and its multi-organ involvement; (2) the etiology, prognosis and treatment of FHF; and (3) use of artificial hepatic support devices. Approximately 50 specialists participated in the conference.

Project Title: CONFERENCE ON GROWTH RETARDATION IN CHILDREN WITH KIDNEY DISEASE

Professional Personnel: Dr. B.T. Burton
Mrs. Billie B. Mackey

Project Description:

This conference was held in Carmel, California from April 22-25, 1977, attended by over 70 participants. Its purpose was to better define the direction for research into the problem of growth retardation and nutrition in children with kidney disease, and assess the factors involved in this disease condition in children. The presentations covered growth measurement during growth inhibition and recovery, energy and protein requirements in children during growth, amino acid and protein metabolism in uremia, role of insulin and somatomedin, bone metabolism and factors affecting bone growth.

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| Project Title: | THE ROLE OF DIETARY FIBER IN HEALTH |
| Amount: | $20,000 (contributed by NIAMDD) |
| Professional Personnel: | Dr. Harold Roth<br>Mrs. Billie B. Mackey<br>Mrs. Elsie C. Yuen |
| Man Years: | Total: .2<br>Professional: .15<br>Others: .05 |

Project Description:

There is an increased interest in the role of dietary fiber in both health and disease. This conference on the Role of Dietary Fiber in Health was held in Bethesda, Maryland on March 28-30, 1977 and supported by several Institutes including NIAMDD, NCI, NHLBI, NICHD, and NIEHS. The conference included a plenary session in which the speakers outlined what is known about fiber in health and disease today. Six simultaneous workshops included presentations of the latest research findings in human gastrointestinal bacteriology, fiber and obesity, fiber and diabetes, fiber and cancer, and fiber effects on lipids and cardiovascular disease. Summaries of the most important points were presented to a final plenary session.

The proceedings of this conference including the summaries and identified research needs will be published in early 1978.

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| Project Title: | CYSTIC FIBROSIS: A COMPREHENSIVE BIBLIOGRAPHY OF THE MEDICAL LITERATURE, 1813-1972 |
| Contract Number: | NIH-NIAMDD-72-2214 |
| Amount: | $8965 |
| Professional Personnel: | Mrs. Billie B. Mackey<br>Mrs. Elsie C. Yuen |
| Man Years: | Total: .15<br>Professional: .1<br>Other: .05 |

Project Description:

Cystic fibrosis, one of the most serious childhood diseases, is diagnosed with increasing frequency throughout the world at a rate of nearly 1000 new cases each year. It claims far more victims as a cause of death than rheumatic fever, diabetes, and poliomyelitis. Cystic fibrosis research at present is directed toward clarifying the clinical manifestations of the disease, uncovering the basic defect responsible, and finding the best approach to treating the disease.

NIAMDD has supported publication of two previous bibliographies prepared by the Cystic Fibrosis Foundation, the first in 1959 and the second in 1966. These unique publications have provided scientists with a valuable tool to better understand, treat, and control cystic fibrosis. The present publication, Cystic Fibrosis: A Comprehensive Bibliography of the Medical Literature, 1813-1972, includes all citations of the relevant scientific literature on cystic fibrosis to 1972. This Bibliography contains a citation list of approximately 5200 titles in the English and non-English languages, and topic and author indices. The indices are designed to help the user locate wanted citations with greater ease.

Project Title: FIRST INTERNATIONAL CONGRESS ON NUTRITION IN RENAL FAILURE

Professional Personnel: Dr. B.T. Burton
Mrs. Billie B. Mackey

Project Description:

This meeting was held in Wurzburg, Germany from May 23-25, 1977, its purpose being to determine the "state of the art" of nutritional problems in renal failure. The program covered relationships between altered metabolism and nutritional requirements, methodologies for assessing nutritional requirements, uses of protein, amino acid and keto acid diets, and parenteral nutrition. The findings are useful in determining future directions for the Artificial Kidney-Chronic Uremia Program.

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| Project Title: | EVALUATION OF HEMODIALYZERS AND DIALYSIS MEMBRANES |
| Contract Number: | NO1 AM-6-2216 |
| Amount: | $30,000 |
| Contractor: | Gulf South Research Institute |
| Project Officer: | Dr. Fernando Villarroel |
| Professional Personnel: | Dr. B.T. Burton<br>Dr. Robert J. Wineman<br>Mrs. Billie B. Mackey |
| Man Years: | Total: .1<br>Professional: .1 |

Project Description:

The Artificial Kidney-Chronic Uremia Program, NIAMDD has been active in sponsoring studies for the improvement of devices and therapy, and has pioneered the establishment of guidelines for the evaluation of hemodialyzers and their components. Two earlier publications in 1970 and 1974 provided guidelines and sources of reference needed by those individuals involved in the development of dialyzers. Advances in this rapidly evolving field required another publication updating these reports. An interdisciplinary study group was formed with members drawn from academic institutions, nonprofit research organizations, industry, and government to develop a definitive publication incorporating the latest technology and research advances in hemodialyzers and dialysis membranes.

Evaluation of Hemodialyzers and Dialysis Membranes was published and distributed by the Artificial Kidney-Chronic Uremia Program, NIAMDD. It is for sale by the Superintendent of Documents, Government Printing Office for a fee.

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| Project Title: | PROCEEDINGS--WHO REGIONAL SYMPOSIUM ON VESICAL CALCULUS DISEASE |
| Professional Personnel: | Dr. G. Donald Whedon<br>Mrs. Billie B. Mackey<br>Mrs. Elsie C. Yuen |
| Man Years: | Total: .25<br>Professional: .15<br>Other: .10 |

Project Description:

The WHO Regional Symposium on Vesical Calculus Disease, sponsored by the World Health Organization and held in Bangkok, Thailand in 1972 was the first international conference devoted to the problem of idiopathic pediatric urinary bladder stone disease. Papers presented at the conference were assembled and published by the Institute as Proceedings--WHO Regional Symposium on Vesical Calculus Disease. The staff provided editorial support in preparing the camera copy and illustrations for printing. Copies have been distributed to a worldwide readership of urologists, pediatricians, clinical researchers, epidemiologists, and others with an interest in some aspect of vesical calculus disease.

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| Project Title: | SYMPOSIUM ON DRUG ACTION AND METABOLISM IN RENAL FAILURE |
| Amount: | $2,500 |
| Professional Personnel: | Dr. B.T. Burton<br>Mrs. Billie B. Mackey |
| Man Years: | Total: .01<br>Professional: .01 |

Project Description:

The Third Rogosin Kidney Center Symposium on Drug Action and Metabolism in Renal Failure was held in New York on October 8 and 9, 1976 under the joint sponsorship of the Rogosin Kidney Center and the Institute.

Patients with kidney disease have an altered metabolism of drugs. The selection and dosage of drugs has to be modified in patients with renal failure due to their altered metabolism. This Symposium presented the most recent information about drug metabolism, active drug metabolites, mechanisms of renal excretion of drugs, drug effects on the kidney, and an analysis of drug selection in the treatment of patients with renal failure.

The Symposium was published in "The American Journal of Medicine," April, 1977 issue and distributed to researchers and clinicians with interests in drug action in renal failure.

Project Title: WORKSHOP ON BILIARY ATRESIA

Professional Personnel: Dr. Sarah Kalser
Mrs. Billie B. Mackey

Project Description:

Approximately 50 medical scientists from the United States and abroad met March 21-23, 1977 in Bethesda, Maryland to address the problem of biliary atresia. The participants sought to define what is currently known about this disease and to examine some provocative concepts regarding the etiology of biliary atresia along immunologic and viral lines.

The developmental anatomy of the liver and the biliary tree, their physiological function in fetal and neonatal life, the immunobiology of fetal and neonatal life, viral diseases of the neonatal liver and use of animal studies were discussed. Intrahepatic and extrahepatic cholestasis and their difficult preoperative evaluation were debated. Differences in operative mortality between the Japanese and U.S. experience in hepatic portoenterostomy were explored, and future exchange in this area was recommended.

Project Title: WORKSHOP ON COLLAGEN METABOLISM IN THE LIVER

Professional Personnel: Dr. Sarah Kalser
Mrs. Billie B. Mackey

Project Description:

As connective tissue collagen increases markedly in chronic liver disease, especially in cirrhosis, some interference in this collagen formation could retard or prevent the transition from chronic active hepatitis to cirrhosis. This workshop, sponsored by the Institute's Liver Diseases Program and the National Institute of Alcohol Abuse and Alcoholism, was held February 28 to March 1, 1977 to highlight areas of needed research. The discussions progressed from the current status of collagen chemistry and immunochemistry to hepatic fibrosis (experimental and human) and comparison of their biochemical, histochemical, immunologic, and cellular features. The effect of alcohol on hepatic fibrosis and the therapeutic inhibition of fibrosis were also covered. Approximately 60 basic research and clinical scientists, and collagen chemists participated in this workshop.

Project Title: WORKSHOP ON VITILIGO

Professional Personnel: Dr. Laurence Miller
Mrs. Billie B. Mackey

Project Description:

A state of the art workshop on vitiligo was conducted by the Institute's Dermatology Program on March 3-4, 1977 at Bethesda, Maryland. It was held to better define the direction of research into vitiligo, a disease characterized by genetically determined patterned loss of melanin pigment following destruction of melanocytes. The meeting agenda covered the magnitude of the problem including its clinical and psycho-social aspects, the pathophysiology and biology of pigment cells (in skin and hair bulbs) and Langerhans cells in vitiligo patients, the immunology of pigment cells, treatment by repigmentation and depigmentation, current research support for vitiligo and recommendations for the future. Approximately 25 participants attended this working meeting.

OFFICE OF THE ASSOCIATE DIRECTOR
FOR
PROGRAM ANALYSIS AND SCIENTIFIC COMMUNICATION

# ARTIFICIAL KIDNEY - CHRONIC UREMIA PROGRAM

Summary Report

## Introduction

The Annual Reports for the previous years provide detailed background on the problems of end-stage kidney disease and elaborate on developments in the modality for its treatment, chronic intermittent hemodialysis. In addition, these reports give a detailed description of the planned and centrally directed contract program of research and development organized at the National Institute of Arthritis and Metabolic Diseases in 1966--The Artificial Kidney - Chronic Uremia Program--which is aimed at development of improved and less expensive dialysis hardware and methodologies and at optimal rehabilitation of chronic dialysis patients. This background material is not repeated here but the reader might wish to familiarize himself with it by perusing the previous reports.

## Current Activities

The Artificial Kidney - Chronic Uremia Program endeavors to bring about improvements in artificial kidney apparatus and methodologies, to increase their effectiveness, to decrease their initial and operational cost, and to improve the rehabilitation of patients treated with chronic intermittent dialysis. Optimal development of new, better dialysis apparatus and methods is still hampered by incomplete knowledge concerning the specifics of the uremic syndrome. Hence, knowledge gained from research into the fundamental aspect of the toxic nature of uremia and clinical studies into the long-term effects of uremia and of chronic intermittent dialysis are critical to arriving at reasonable solutions to the overall problem. Thus, the Program not only sponsors hardware improvement per se, but supports a wide spectrum of research ranging from studies designed to clarify the toxic nature of uremia and clinical studies on the complications of chronic uremia and maintenance dialysis, to development of improved dialysis or apparatus.

At present about 70 research and development projects are supported by contracts with universities, nonprofit and industrial research laboratories to give a broad approach to problems of highest priority. Projects are focused on problem areas including clinical trials of more efficient or less costly dialyzers, development of special adsorbents for uremic toxins, and studies to elucidate the clinical consequences of the aberrant metabolic changes in uremia.

The Program is also funding studies holding the promise of novel new treatments such as "hemofiltration" as well as those which might lead to significant improvements in the efficacy of hemodialysis and peritoneal dialysis methodology. At the same time, efforts are being directed toward amelioration of the clinical complications of treatment. Priority is currently being given to studies related to the anemia of uremia, prevention of uremic osteodystrophy (diminished bone density), the peripheral and central nervous system deterioration of chronic renal failure on long-term maintenance treatment, and impaired sexual function in dialysis patients.

## ACCOMPLISHMENTS

### Accelerated Atherosclerosis in Hemodialysis Patients

The leading cause of death among patients undergoing chronic hemodialysis is cardiovascular disease. This is the most important factor limiting survival. Furthermore, even in renal patients receiving a successful kidney transplant accelerated therogenesis (development of "hardening" of larger arteries) occurs frequently. Several studies are beginning to characterize some of these abnormalities. Dr. Robert Swenson at Stanford University has shown that dietary adjustment can have beneficial effects in lowering plasma triglyceride levels. By substituting a diet in which fat replaces a portion of the previously used carbohydrates, mean fasting triglyceride levels were lowered in dialysis patients from 195 mg % to 154 mg %. Further study of these factors is required, but changes in dietary recommendation for dialysis patients may be in order. Currently, in most such diets about 50% of the calories are derived from carbohydrates and about 35-40% from fats. Dr. Swenson's study diet which resulted in lower triglycerides had 55% of the calories derived from fat.

### Greater Role for Improved Peritoneal Dialysis

The Institute has placed considerable emphasis on further research and development in the field of peritoneal dialysis, an alternate mode of dialysis which has been known for many years but which has not been used frequently in the past because of its high cost and time requirements. This situation has now changed completely. Research sponsored by the Institute has resulted in the development of a permanent access device to the peritoneal (abdominal) cavity (the implantable Tenckhoff catheter) which permits relatively simple and repeated access for purposes of peritoneal dialysis. Subsequently, fully automated, closed-cycle peritoneal dialysis machines were developed which can be used in a center setting or in a patient's home for maintenance peritoneal dialysis at a considerably lower cost than before.

During the past year, while some investigators have demonstrated the feasibility and relatively low cost of home peritoneal dialysis,

others, to avoid the long 10-hour treatment sessions, have demonstrated the feasibility and efficacy of daily peritoneal dialysis in the evening, with and without automated dialysate delivery systems. Using today's techniques, the rate of peritonitis due to microbial contamination has been reduced considerably and has been reported to be as low as 0.2% in daily self-dialysis. Also, in peritoneal dialysis, the handling of dialysis access is simpler, less critical and, in home dialysis, in case of a mishap, less fraught with serious risk. One should also mention that peritoneal dialysis is invaluable for special groups, such as patients with serious blood access problems, or with severe cardiovascular disease, for those of advanced age, and for diabetic patients in whom it does not appear to induce as rapid a loss of vision. Moreover, because it is simpler to learn and perform than is home hemodialysis, it may be used by patients who live alone who are otherwise not eligible for home dialysis.

Dr. Popovich and associates at the University of Texas have discovered a potentially major and revoluntionary improvement in the conduct of peritoneal dialysis. He has shown that use of 10 liters of dialysate fluid per day is adequate to maintain the average adult patient. The technique involves essentially an approximation of continuous 24 hour/day treatment. Two liters of dialysate are infused intraperitoneally via an indwelling catheter. The patient conducts his normal activities for four hours, after which the dialysate is drained and another two liters instilled. The procedure is repeated five times per day. Limited clinical experience in two centers has shown this mode of therapy to be most promising. Further evaluation is currently a high priority program objective.

Reduction in Adverse Patient Symptomatology Associated with Dialysis

Various aspects of acid-base control and buffers for use in dialysate fluid are under investigation. Dr. Scribner and associates at the University of Washington have reported a remarkable improvement in dialysis related patient symptomatology when bicarbonate is used as the buffer in dialysate fluid instead of the traditional sodium acetate. Marked reductions in the incidence of nausea, vomiting, headache, tiredness, dizziness and hypotension occurred when bicarbonate buffer was used for large surface area rapid dialysis. Additionally, patients were able to tolerate much higher rates of ultrafiltration (fluid removal).

Dr. Jonas Bergström and associates have also shown major improvements in the ability of patients to tolerate high rates of fluid

removal. He has pioneered a sequential process of hemofiltration for the first hour of treatment followed by conventional dialysis. Problem patients exhibit a much lower frequency of hypotensive episodes under the new regimen than conventional hemodialysis with simultaneous ultrafiltration.

Contractor: Baylor College of Medicine

Amount: $85,167

Title: Abnormalities of Carbohydrate Metabolism in Chronic Uremia.

Objectives: The objectives of the research performed in this contract are to establish a precise definition of glucose and glucose precursor kinetics, and an elucidation of their regulation in chronic uremia. Major attention is being directed toward three discreet aspects of carbohydrate metabolism. These include a characterization of the role of inappropriate and excessive gluconeogenesis in the hyperglycemia of uremia, the impact of uremia on the regulation of muscle amino acid and protein metabolism and the influence of uremia on the suppressibility of endogenous hepatic glucose production by exogenous glucose as in dialysate solutions.

Major Findings: Preliminary results show that glucose production+ is increased 94.6 $\pm$ 12.2% in nondialyzed uremic patients, and 47.9 $\pm$ 7.6% in patients on hemodialysis compared to matched controls. Alanine production was increased 47.9 $\pm$ 6.1% and 53.4 $\pm$ 3.9% respectively. Alanine used for gluconeogenesis was increased 96.2 $\pm$ 10.2% in uremia, and the relative percentage of alanine used by gluconeogenesis increased from 28.4 $\pm$ 4.9% in controls to 42.2 $\pm$ 5.8% in uremia. Rates of glucose produced from alanine and the percentage of total glucose production derived from alanine were increased similarly. Parathyroid hormone in vitro increased skeletal muscle alanine and glutamine formation and release, owing to increased rate of muscle proteolysis. Protein degradation in alanine and glutamine formation in muscles from uremic rats were increased relative to controls and no further increase was produced by parathyroid hormone. Parathyroid hormone action on muscle proteolysis was not accompanied by an increment in cyclic AMP, or by activation of adenylate cyclase.

Proposed Course: Completion of ongoing studies of glucose and alanine kinetics and precursor-product interrelationships in patients with chronic uremia is expected. Patients known to be diabetic prior to the development of renal disease will be compared to patients without prior chemical diabetes or personal or family history of diabetes. Studies will be performed to determine the acute impact of dialysis itself on glucose and alanine kinetics and precursor-product inter-relationships in uremic subjects. Simultaneous isotope infusion studies will be performed prior to their first dialysis of the week and immediately after the performance of their last dialysis of the week. The metabolic basis

for the acquired in sensitivity to epinepherine or serotonin suppression of protein degradation will be investigated using specific kinetic assays for adenylate cyclase. These studies will permit localization of the defect in uremic skeletal muscle cyclic AMP generation to either a failure of binding of adequate quantities of effector due to decreased receptor concentrations or an alteration in effector-receptor binding kinetic or to intrinsic abnormalities of skeletal muscle adenylate cyclase. Using dual label techniques, the influence of exogenous glucose load such as dialysate on endogenous glucose production rates will be determined in normal volunteers and in patients with chronic uremia. Determinations will be possible of the rates of the glucose transfer between the patient and the dialysate solution and the influence of dialysate glucose on the production and utilization of glucose in vivo.

These research approaches will provide a complete evaluation of the molecular and physiologic basis for the abnormal carbohydrate metabolism of uremia, thereby developing mechanisms for rational therapeutic intervention.

Contractor: University of California at San Francisco

Amount: $74,190

Title: Mechanism of Hyperlipidemia in Chronic Renal Failure.

Objectives: The association between chronic renal failure and severe hyperlipidemia has been recognized for many years. Vascular disease of the atherosclerotic type is hastened by chronic uremia and there appears to be an abnormality in the transport of fat. Observations in the investigator's laboratory has led to an hypothesis that there may be an important connection between the plasma level of high density lipoproteins. The investigator has shown that HDL is the primary carrier of "activator" peptides which are essential to the hydrolysis reaction catalyzed by lipoprotein lipase. The objective of the research proposal is to perform detailed analysis of the lipoprotein spectrum of patients with renal disease and to further study the content of each of the approprtein of HDL in VLDL.

Major Findings: The analysis of the lipoprtein spectrum of uremic patients has revealed interesting correlations. Fasting plasma samples taken immediately before heparinazation and dialysis were compared with fasting plasma samples taken from normal control subjects. The results are as follows: Very low density lipoprotein (VLDL) triglycerides were significantly elevated in anephric patients ($P < .001$). Low density lipoprotein (LDL) cholesterol was significantly lower in the patients ($P < .01$). Both high density lipoprotein (HDL) cholesterol and protein were markedly lower in the patients ($P < .001$). There was an excellent negative correlation between the level of HDL cholesterol and HDL protein, and the VLDL triglycerides. Since the HDL serve as an activator for VLDL removal by lipoprotein lipase, and since LDL is a direct product of VLDL metabolism, these data clearly point to a defect in the removal mechanism for VLDL. Preliminary studies have shown that the composition of HDL is not different from normal patients although the plasma content is lower in patients with end stage renal disease. The VLDL, however, show quite different results. The ratio of activator peptides to inhibitor peptides is markedly lower in the patients so far studied. This ratio shift may have a marked effect on the lipoprotein lipase reaction.

Proposed Work: Detailed analyses of the lipoprotein spectrum will be carried out on the following groups:

1. Anephric patients on dialysis.
2. Patients with glomerulonephritis on dialysis. for varying periods of time.
3. Patients with polycystic kidney diseases.
4. Normal control subjects.

Detailed characterization of each of the lipoprotein of HDL and VLDL will be carried out. Particular emphasis will be placed on the ratio between Apo C-II, the activator peptide for lipoprotein lipase and Apo C-III, the inhibitor peptide for LPL. A detailed study will be carried out of the activator or inhibitor potential of whole plasma lipoprotein and apoprotein from VLDL and HDL. Lipoprotein lipase from raw milk will be used as the enzymes source and Intralipid will be used as a substrate. Correlations will be sought between the enzyme reaction rate and the ratio of C-II over C-III peptides. Finally, the possibility of therapy with C-II peptide obtained from human plasma will be explored.

Contractor: University of California, San Francisco

Amount: $48,516

Title: Evaluation of Chronic Peritoneal Dialysis in Children: Comparison with Hemodialysis.

Objectives: To compare the efficacy of home peritoneal dialysis in children with standard center-based hemodialysis.

Major Findings: Chronic peritoneal dialysis was initiated in 2 children and chronic hemodialysis was initiated in 4 children. Peritoneal BUN clearance was 6.6 ml/minute in 11 kg child was 20 ml/minute in a 36 kg child. Once a week in-hospital dialysis for 48 hours was satisfactory in the first patient. Three times weekly, 12 hour overnight home dialysis was satisfactory in the second patient. There were 5 patient months of peritoneal dialysis and 17 patient months of hemodialysis. There were no deaths. The major problems encountered were: 1. Catheter complications in peritoneal dialysis patients and shunt and fistula complications in hemodialysis patients, 2. Renal osteodystrophy, retarded growth and sexual development, 3. Poor psychosocial adaptation in 2 hemodialysis patients. The costs of treatment were high in both groups.

Proposed Course: The studies will be continued. Parameters to be followed and compared include survival, patient acceptance, dialysis access, biochemical control, patient growth rate, nutrition, sexual maturation, psycho-social adaptation and cost.

Contractor: University of California, Los Angeles

Amount: $112,964

Title: Nitrogen Metabolism in Uremia

Objectives: To study the metabolism of amino acids and their products and the enzymes which mediate their metabolism in uremia.

Major Findings: Further characterization of the daily histidine requirement has been accomplished. It has also been shown that in chronically uremic patients, diets providing 22 gm of high quality protein and 21 gm of essential amino acids appear to promote neutral or positive nitrogen balance with relatively low rates of urea production. Interrelationships between protein intake and plasma amino acid levels were evaluated and results indicate that in uremic and hemodialysis patients, uremia *per se* and low protein diets each cause certain abnormal plasma amino acid levels.

Proposed Course: Ongoing studies will be continued. Patients with acute renal failure requiring hemodialysis will be evaluated to determine the metabolic protein, amino acid and caloric requirements to provide adequate nutrition.

Contractor: University of California at Los Angeles

Amount: $103,503

Title: Controlled Evaluation of Maintenance Peritoneal Dialysis

Objective: To evaluate the effect of long-term peritoneal dialysis (PD) on nutritional status by: a) assessing nutritional state in uremic patients randomly assigned to treatment with either PD or hemodialysis (HD) as in a VA Cooperative Study; b) measuring kinetics of albumin loss during PD and studying the efect of such loss on albumin metabolism; c) measuring plasma proteins specifically those that may appear in effluent dialysate and assessing the effect of such losses; and d) defining protein requirements in patients undergoing maintenance PD.

Study effectiveness of long-term PD in uremic patients with diabetic nephropathy. They will: a) compare results in diabetic patients randomly assigned to treatment with either HD or PD in a parallel VA Cooperative Dialysis Study; b) evaluate whether microvascular disease effectively reduces the permeance and clearance of the peritoneal membrane in diabetic patients on PD; and c) evaluate whether intraperitoneal insulin may be an effective method for managing hyperglycemia that occurs in diabetic patients during PD.

Major Findings: Studies were initiated to 1) evaluate nutritional status of patients on regular peritoneal dialysis (PD) and 2) study efficacy of regular PD for the management of patients with end-stage diabetic nephropathy. In a VA Interagency Agreement, the nutritional status of patients randomly assigned to either self-care hemodialysis (HD) or self-care PD is being assessed as part of a VA Cooperative Study. In a parallel study, patients with end-stage diabetic nephropathy are randomly assigned to HD or PD in 8 participating VA hospitals. These 2 studies are just underway.

Loses of plasma proteins, including immunoglobulins, into effluent peritoneal dialysate have been studied, and the kinetics of albumin metabolism is being evaluated in patients treated with maintenance PD. Studies in dogs suggest possible efficacy of intra-peritoneal insulin for managing hyperglycemia in diabetic patients receiving PD; studies in humans are underway. The permeability in clearances across the peritoneal membrane of uremic patients with and without diabetes were compared; initial data suggest comparable rates of peritoneal clearance and permeance.

Proposed Course: This project will be continued.

Contractor: University of California at San Diego

Amount: $111,074

Title: Blood Pressure Control with Hemodiafiltration

Objectives: Evaluation of the efficacy of hemodiafiltration in management of hypertension in chronic uremia. Hemofiltration is being compared to standard hemodialysis to control hypertension.

Major Findings: Three patients have been studied completely. So far data points away from simple sodium/volume reduction as an explanation for improved blood pressure control and supports the presence of an autonomic nervous system dysfunction as the cause for hypertension that responds to hemofiltration. This information has been derived from a study protocol designed in a 3 month "Switchback" format (i.e., A, B, A'). Four patients have hypertension and one is normotensive "tone control". Studies of blood pressure weight, weight gain between treatments, total body sodium, extracellular fluid volume, plasma resin, plasma dopamine B hydroxylase, serum electrolytes, BUN, creatinine, uric acid, and power spectrum analysis at the EEG have been completed (A, B, A') for the first three patients and underway for the next two. (A and A, B completed).

Proposed Course: Completion of the 15 patients initially proposed to study to better define the effectiveness of hemodiafiltration as an adjunctive therapy for the treatment of hypertension associated with end-stage renal failure.

Contractor: Cedars-Sinai Medical Center

Amount: $131,056

Title: Sorbent-based Regenerative Peritoneal Dialysis System

Objectives: To develop a peritoneal dialysis system in which the dialysate is circulated through a sorbent cartridge for regeneration.

Major Finding: Further progress has been achieved on the sorbent regeneration of peritoneal dialysate. Human subjects are now being dialyzed successfully for periods of up to twelve (12) hours with biochemical results identical to those obtained with standard dialysis techniques. Particulate matter contamination does not occur, however, episodes of peritonitis have occurred in these clinical subjects. These appear to be due to procedural techniques in the conduct of clinical experiments and not due to contamination of the components of the system. The procedural difficulties have been changed and it is estimated that the maintenance of sterility will no longer be a problem. Aside from the episodes of peritonitis, the clinical subjects have tolerated the procedure well. Animal studies have been continued and show the potential for increasing dialysis efficiency by 100% or more using techniques of continuous flow peritoneal dialysis.

Proposed Course: Two patients will be maintained with home peritoneal dialysis using two automatic, sorbent type, dialysate delivery systems. Patient outcome will closely monitored.

Contractor: Celanese Research Company

Amount: $100,000

Title: Preparation and Evaluation of Optimized Hemodialysis Membranes

Objectives:

1. Fabricate cellulose acetate membranes CA-1 and CA-2 to maintain a supply of each (ca. 5000 linear feet) adequate for ongoing clinical investigations.

2. Develop practical techniques for the fabrication of the following experimental membranes from cellulose acetate and supply each to interested clinical investigators as required:

   (a) A membrane with a sharp molecular weight cutoff in the 800-1000 region but with hydraulic and small molecule permeabilities similar to Cuprophan.

   (b) A high flux membrane with hydraulic permeability similar to Rhodia PAN membrane (20-30X Cuprophan) but with significantly higher solute permeabilities.

   (c) Thinner versions of CA-1 and CA-2 with thicknesses of 0.8-0.9 mils and possessing higher solute permeabilities without increased hydraulic permeability.

3. On a limited basis, continue to explore promising new concepts relevant to the fabrication of cellulose acetate dialysis membranes, particularly glycerinated versions of CA-1 and CA-2.

Major Findings: Quantities of cellulose acetate membranes CA-1 and CA-2 were fabricated on the continuous casting machine in 16-inch width. These quantities are adequate for clinical and laboratory studies. Repeated attempts to transfer, to a practical large scale, casting procedures for a low molecular weight cutoff membrane have been unsuccessful so far. Solvent exchange techniques on preformed membranes as well as film casting to produce the target membrane properties were tried after being demonstrated on a small scale. The direct casting approach is still promising and will be pursued further. A feasible procedure to produce a "dry", glycerinized membrane was demonstrated in the laboratory. The "dry" membrane exhibited good retention of transport properties in storage. A thinner version of CA-1 was produced in quanity on the continuous casting machine, although some difficulty was experienced in trying to reach thicknesses of 0.8-0.9 mils. A thinner version of CA-2 will be prepared. A very high flux CA membrane with a water permeability (25 x Cuprophan PT-150) similar to Rhone-Poulenc's PAN membrane was fabricated in quantity on the continuous casting machine. In response to membrane quality problems noted by clinical investigators, a number of corrective measures were taken to improve membrane quality.

Proposed Course: This study will be concluded at the end of the present year with the exception of supply of needed experimental membrane to clinical investigators.

Contractor: University of Colorado

Amount: $121,200

Title: Trace Element Abnormalities in Chronic Uremia

Objectives: To determine the levels of concentration of trace elements in dialyzed uremic patients; to determine the clinical importance of trace element abnormalities, and to establish the concentration of trace elements required in the dialysate solution.

Major Findings: Both X-ray fluorescence and atomic absorption spectometry methods have been developed for measuring trace elements. The program of characterizing the tissue trace element abnormalities associated with chronic uremia and with dialysis has continued, with emphasis on obtaining better measurements, larger sample populations, additional tissues and additional elements. This effort has provided the basic reconnaissance information regarding the trace elements which are disturbed and the tissues in which those disturbances occur. Major findings include an association of aluminum with a fatal neurological syndrome, and uranium in the tissues of some dialyzed uremics.

Proposed Course: Tissue trace element concentration will continue to be measured in normal individuals who have experienced sudden death and in uremic patients who have died while being treated with hemodialysis or peritoneal dialysis. Several geographic areas will be considered.

Urine, plasma and hair trace element measurements will be performed in uremic patients to determine the effect of duration of uremia on trace element composition. These measurements will also be performed after transplant.

Contractor: University of Connecticut

Amount: $86,883

Title: Peritoneal Permeability and Drug Enhancement in Uremia

Objective: Comparison of clearance values of solutes of varied size before and after vaso-dilator enhancement will allow characterization of peritoneal permeability, will quantify peritoneal clearances of numerous drugs, and will identify quantitatively the beneficial effect of pharmacologic manipulations.

Major Findings: Because the Food and Drug Administration approval has not been granted for administration of standard drugs for an unapproved purpose, the study has been limited mostly to determination of baseline clearances. The first year of study of peritoneal permeability has expanded the observations that peritoneal clearances of urea 19.3 and creatinine 14.5 m;/min decrease with vascular disease to 13.9 and 8.3, consistent with decreased permeability, a phenomenon that we demonstrated is partially reversed by dipyridamole given orally. Preliminary data also suggest that furosemide given systemically, increases peritoneal permeability.Digoxin clearance when corrected for protein binding also reflects peritoneal permeability decreasing from 9.2 to 4.1 ml/min with vascular disease and increasing to 5.5 ml/min after dipyridamole. Clearances of fatty acids also appear to reflect peritoneal permeability. Values are frequently so high as to suggest that peritoneal dialysate equilibrates with a fluid of different composition than peripheral plasma. In the rabbit, aminophylline intravenously or intraperitoneally had no salutary effect on clearances while intraperitoneal isoproterenol significantly ($p < 0.01$) increased clearances of urea and creatinine. Preliminary data suggest that furosemide may increase net transport of water and sodium during hypertonic peritoneal dialysis.

Proposed Work:

1. To determine the effect on permeability of the peritoneum, as reflected by clearances of solutes of different size in humans of specific drugs given systemically or intraperitoneally.
2. To measure the peritoneal clearance of drugs of clinical importance in management of renal failure.
3. To continue studies of fatty acid clearance by peritoneal dialysis, with a view toward identifying whether peritoneal dialysate equilibrates with fluids of different composition than peripheral plasma, and
4. To extend these studies by peritoneal dialyses in rabbits, focusing on characterization of the most effective and safest drugs for increasing peritoneal permeability.

Contractor: Dalton Research Center

Amount: $54,625

Title: Effects of Peritoneal Dialysis Solutions on the Microcirculation of the Rat and on Peritoneal Clearances in Man.

Objectives: To evaluate the efficacy of and to determine the mechanisms by which alterations in the physical and chemical composition of peritoneal dialysis solutions may increase solute clearances for peritoneal dialysis. This will be accomplished by coordinating, complementing and correlating protocols for clinical studies of peritoneal dialysis with those for in vivo animal studies of the microcirculation. In particular, a relationship between the efficacy of peritoneal solute transport and the vasoactivity of different peritoneal dialysis solutions is being sought. This will be accomplished by comparing the vasoactivity of dialysis solutions in an in vivo animal model with the peritoneal clearances in man. Specifically, the effects of 1) adjustment of the pH of commercial dialysis solutions, 2) the addition of nitroprusside to commercial dialysis solutions at pH 5.5 and 3) the addition of nitroprusside to commercial dialysis solutions which have been adjusted to pH 7.4 will be studied. The minimum dose of nitroprusside per liter of dialysis solution which is required for maximal vasodilatation of the microcirculation and for maximal increases in clearances during clinical dialysis will be determined. The relationship between the vasodilatory properties of nitroprusside and the effect of nitroprusside on solute clearances in clinical dialysis will be determined and the anatomical location for the vasodilatory action will be determined from microcirculatory studies.

Vasodilatory properties of nitroprusside will be studied in dialysis solutions before installation into the human peritoneum and after damage and will be related to concentrations of nitroprusside, thiocyanate and cyanide.

Results: Pending.

Proposed Work: Above.

CONTRACTOR: Donald Dexter Van Slyke Memorial Foundation, Inc.

AMOUNT: $130,504

TITLE: Gastrointestinal Dialysis as an Adjunct to Therapy of Renal Failure

OBJECTIVE: To investigate the oral perfusion of the gastrointestinal tract (as a dialyzing tube) to remove uremic toxins accumulated in the blood of patients as a means of treatment of renal failure.

MAJOR FINDINGS: Through the spontaneous drinking by subjects of isosmotic saline solutions containing 180-220 millimolar mannitol for about 3 hours, 54 whole-gut perfusions were performed in 22 patients with chronic uremia. Average amounts of nonprotein nitrogen, urea nitrogen and creatinine excreted in the diarrheal fluids were 4556, 3069 and 184 mg per perfusion study. The intestinal clearances of urea and creatinine during perfusion were found to be 27.1 and 6.9 ml/minute respectively. The rates of intestinal excretion of nonprotein nitrogen and urea nitrogen were proportionate to the initial blood urea nitrogen levels with correlation coefficients of 0.926 and 0.907 respectively. After whole-gut perfusion, the blood urea nitrogen was decreased by 11.5 mg/100 ml ($P < 0.001$) and plasma creatinine decreased by 0.37 mg/100 ml ($P < 0.001$). There was no retention of perfusion fluid, as evidenced by a significant decrease of 380 grams body weight after perfusion ($P < 0.001$). Conclusions are that whole-gut perfusion can remove considerable amounts of urea, but relatively less creatinine, in chronic uremic patients.

PROPOSED COURSE: This work will continue with emphasis on clinical application in uremic patients with glomerular filtration rates between 3 and 5 milliliters per minute to ascertain the precise applicability of gastrointestinal dialysis in end-stage renal disease.

Contractor: Exxon Research Engineering Company

Amount: $139,933

Title: Liquid Membrane Capsule System for the Treatment of Chronic Uremia

Objectives: To develop a liquid membrane to encapsulate reactants to be used as toxin traps in the treatment of chronic uremia.

Major Finding: The effort is concentrated on the urea which is removed by the LMC in the form of ammonia. Success of this system is based on tolerance and palatability, effective ammonia uptake and effective urease concentration in the lumen. Two chronically uremic dogs volutarily consumed a meat broth - LMC (citric acid reagent) suspension for six days. No vomiting, diarrhea or signs of intolerance were observed. Urease has been encapsulated in LMC and released *in vitro* into a synthetic intestinal fluid containing bile and pancreatin. Stabilized LMC effective in removing ammonia have been prepared and undergo little physical change in over 90 minutes under *in vitro* simulated severe intestinal conditions. LMC have shown capacity (*in vitro*) for methylguanidine, another uremic toxin.

Proposed Course: The overall goal of this year's contract will be to produce clinically useful liquid membrane capsule suspension. Work will continue in the following areas:

(a) Metabolic studies in uremic dogs:
- Determination of optimal urease dosage and LMC clearance
- Long term efficacy

(b) Preparation of a medical grade polyamide strengthening agent for future human consumption.

(c) Preparation of a urease releasing LMC

(d) Improvement of the LMC formulation to provide longer life to the capsule

Contractor: Franklin Hospital

Amount: $108,000

Title: Solute Kinetics in Intermittant Dialysis Therapy

Objectives: The object of the studies is to investigate the mechanisms that control the concentrations of various solutes found in the chronic uremic patient and how they can be related to the patient's state of health. This year, proposed work concentrates on the kinetics of acid-base metabolism. One of this year's objectives is to obtain data to compare normalization of acid-base balance with use of acetate and bicarbonate dialysate buffers. Additionally, the relative clinical effectiveness of acetate and bicarbonate dialysate will be compared over a long (6 month+) time period.

Major Findings: Briefly, the contract work has consisted of: (1) Establishment of precise methods to measure the bicarbonate $CO_2$ system, (2) Design and development of an online bicarbonate proportioning system, (3) In vitro and in vivo determination of bicarbonate transport with the proportioning system, (4) Short and long term kinetic analysis of acid-base balance with bicarbonate dialysis therapy, (5) Development of a kinetic model to describe the data obtained and provide values for bicarbonate flux rates, the rate of alkalinization of intracellular water and bone buffers and cumulative values for bicarbonate flux.

Additional nitrogen balance studies in the Metabolic Ward at the U.C. Renal Center verified the linear relationship between net urea generation rate ($G_U$) and protein catabolic rate (PCR). Utilization of glucose in the dialysate kept the patient in positive nitrogen balance post dialysis.

Proposed Course: Current plans are to continue this definitive investigation.

CONTRACTOR: University of Goteborg

AMOUNT: $47,056

TITLE: Compact, Low-Volume, Self-Regenerating Artificial Kidney

OBJECTIVE: Development, in vitro and animal evaluation of a compact artificial kidney with emphasis on performance of the urea adsorption resins.

MAJOR FINDINGS: A new design of the compact dialyzer has been constructed. It has a single container housing both coil, regenerating cartridge and dialysate solution. These parts are placed on top of a unit containing pumps, electronics and safety devices. Increasing the volume of the resin-charcoal containing cartridge by 20-30% was found to improve performance.

Of resins tested as urea-sorbent, one highly crosslinked sulfonated polystyrene compound in hydrogen form was found to be satisfactory. In vitro and in vivo tests, the latter on dogs, were performed with respect to pretreatment with different cations, the main being $Ca^{2+}$. The aim was set to remove 40 g of urea from 5 liters of "serum" solution by means of the urea sorbent mixed with activated charcoal in the dialyzing unit. One conclusion can be made from these results. The resin mixed with charcoal in the cylindrical cartridge constitutes a 11 mm thick layer with a surface of 440 $cm^2$. This arrangement works well as an urea adsorber, and less well for ion exchange, which is an advantage.

PROPOSED COURSE: Portions of this project concerning the urea adsorption resins will be continued.

Contractor: Gulf South Research Institute

Amount: $115,607

Title: Membranes and Materials Evaluation: Permeabilities Physical and Mechanical Properties

Major Finding:

- New Marker Solutes: A set of four decapeptides has been synthesized and purified and tagged. Their peculiarities in several membranes is being measured.

- Trace Elements: Spark source mass spectroscopy has been used to establish trace metal content of cuprophan.

- Mass Transfer: Rejection coefficients for a number of solutes in three membranes has been determined in two different experimental cells.

- Dialyzer Characterization Work: On mass transfer characterization of dialyzers under different ultrafiltration rates has been initiated.

Proposed Course:

Work will continue in the following areas:

1) Transport of trace metal ions through dialysis membranes. Binding constants for several protein and metal ion concentrations will be determined.

2) Mechanical and permeation properties of new membranes determination.

3) Membrane rejection studies.

4) Mass transfer characterization of hemodialyzers with ultrafiltration.

5) Validation recommended test protocols.

6) Evaluation commercially available hemosorption devices.

7) Evaluation hemofiltration devices.

8) Development of a new method to determine preselectivity of ultrafiltration membranes using polydisperoid dextrans.

Contractor: Harbor General

Amount: $165,000

Title: The Role of Acetate in Dialysate for Hemodialysis

Objective: Establishment of analytic methods for acetate, cholesterol, cholesterol esters, free fatty acids, phospholipids and triglycerides were developed. An apparatus for continuous measurement of $^{14}CO_2$ in expired air was constructed. The techniques were employed to study acetate metabolism during dialysis in humans and in dogs. The main studies determined the metabolic fate of acetate-1-14C delivered during hemodialysis in uremic (bilaterally nephrectomized) dogs. Nearly 90% of the acetate delivered and retained in uremic dogs during dialysis is converted to $CO_2$ by metabolism via the tricarboxylic acid cycle.

Major Findings: The results of these studies indicated that 83.7% of the infused acetate-1-14C was metabolized to $^{14}CO_2$ by 8 hours post dialysis. An additional 5.4% of the infused acetate was lost to dialysate. The remaining 11.9% of the unrecovered radioactivity was still present in the animals as lipids $^{14}CO_3^-$, $CO_2$ and other products of acetate metabolism. Small but significant amounts of 14C were found to be incorporated into blood and liver lipids. No major differences in the deposition of acetate was observed in uremic vs. nephrectomized nonuremic dogs. Acetate T 1/2 and metabolic clearance rate was the same in the uremic and nonuremic dogs, acetate disappearance from the blood followed first order kinetics. During the infusion blood lipids remained constant except for free fatty acids which increased significantly. Serum pyruvate also increased significantly during the infusion.

Proposed Work: Similar studies will be performed in humans to determine the metabolic fate of acetate in hemodialysis patients. The exact amount of acetate converted to $CO_2$ via the TCA cycle will be ascertained by infusion of acetate-1-C14 and measurement of $^{14}CO_2$ expired and concentration of intermediary metabolites in this process.

A group of patients with diabetes and hepatic disease will be studied to determine how these factors alter acetate intolerance.

Uremic dogs maintained on hemodialysis will be studied in a similar pattern for a period of 2 weeks. Another group of parathyroidectomized dogs will be studied to elucidate the role of the parathyroids in acetate metabolism.

Metabolic consequences of dialysis with acetate will be determined by comparison blood lipids in patients dialyzed on acetate and then bicarbonate for several weeks. After termination of bicarbonate therapy the patient will be returned to acetate containing dialysate and restudied to determine changes in lipid production.

Contractor: Harvard University

Amount: Estimated $700,000

Title: National Cooperative Dialysis Study

Objective: The overall objectives are to develop quantitative guidelines for administering dialysis therapy. The study is designed to compare four experimental therapies for relative response. The range of each dialysis episode will be grouped around two treatment levels for blood urea nitrogen (BUN), pre-dialysis levels of 60-80 and of 100-120. The duration of each dialysis will be individualized for each patient so that the pre-dialysis solute concentration levels are held at one of the two above ranges. The duration of each dialysis, however, will be modified somewhat so that one-half of the patients will be treated with shortened dialysis ($3 \pm 1$ hours) and compared to longer dialysis ($6 \pm 1$ hours). A higher molecular weight solute will be measured as well, but not controlled as will be the pre-dialysis BUN. Patient response will be measured by a variety of parameters.

Proposed Course: This project will continue into the full study phase.

Contractor: University of Kansas Medical School

Amount: $74,215

Title: Quantitation of Response to Erythropoietin in Chronic Uremic and Anemic Mice with Polycystic Kidney Disease

Objective: The purpose of this study is to examine whether these chronic uremic mice represent a suitable model for the assessment of the therapeutic efficacy of erythropoietin in the treatment of the anemia of uremia. Three types of experiments are to be carried out: 1) Uremic and nonuremic mice are made polycythemic by i.p. infusion of isogeneic red cells in order to suppress endogenous Epo. The erythropoietic response to a single dose of Epo. is then measured by means of $Fe^{59}$ incorporation in RBC's and by reticulocyte counts. 2) Anemic mice are given daily injections of Epo. and their reticulocytes, hematocrit and BUN are determined at weekly intervals, 3) As the first step in the assessment of the effect of erythropoietin on the number of morphologically unrecognizable erythyroid percursor cells (CFU - E) in the uremic mice, the number of CFU-E in the femoral bone marrow will be measured by the in vitro plasma clot culture method. This will be compared with those found in non-uremic mice.

Results: Preliminary results so far mice with BUN's above 50 mg % required 2-4 times the dose of Epo to elicit a commensurate erythropoietin response as found in non-uremic controls. In one group of uremic mice (BUN 42-73 mg %) daily injections of 2 units of Epo raised the reticulocyte counts significantly but resulted in only small hematocrit rise within a three week period. After raising the Epo dose to 5 units/day hematocrits rose to the normal range within 3 weeks. Finally in 8 uremic mice the number of femoral CFU-E was found to be in the normal range.

Proposed Work: Above.

Contractor: Karolinska Institutet

Amount: $92,543

Title: A Study of Uremic Toxicology

Objectives: Utilizing the technique of high speed gel filtration followed by gradient elution, uremic body fluids have been separated into 7-9 different fractions in the middle molecule range (7a, b, c, d, etc). The investigators plan to further purify and chemically define the uremic middle molecule fractions. Screening of uremic patients undergoing various forms of treatment will continue in order to assess the clinical value of middle molecule determination. The effect of various dialysis schedules on the accumulation of middle molecules will be investigated. The performance of various dialyzers and other extra-corporeal devices with regard to middle molecule removal will be assessed. Studies of the in vitro toxicity will be continued with highly purified middle molecule fractions.

Major Findings: The methods for separation and quantitation of middle molecules in biological fluids, i.e., high-speed gel filtration combined with gradient ion exchange chromatography were developed into an automatic analytical system with recording and storage of data using computer techniques. Middle molecule material from uremic plasma and urine was separated and desalted by preparative chromatographic methods and analyzed by isotachophoresis. This enabled the estimation of the concentration of fraction 7c on a weight basis. Most patients with pericarditis had at least one high middle molecule peak in plasma. In patients treated with diet, reduction in protein intake was associated with an increase in some peaks and a decrease in 7c. In hemodialysis patients, infection was associated with an increase in 7c plasma. Considerable variation in estimated middle molecule generation rates were found in different uremic patients. Ultrafiltration only (without simultaneous diffusion) resulted in a decrease in peak 7b and c. In rats with renal failure middle molecules were found similar to those of uremic man. Peak 7 material inhibited erythrocyte $^{59}Fe$ incorporation in mice.

Proposed Course: This project will be continued.

Contractor: Leland Stanford Junior University

Amount: $128,200

Title: The Role of Acetate in Dialysate for Hemodialysis.

Objective: The major goal has been to develop a sensitive and reproducible assay for plasma acetate. This has been accomplished. In conjunction, metabolic balance studies of $14_C$ acetate during dialysis have been planned and begun in order to evaluate pathways of acetate metabolism. The dialysance of acetate has been quantitated and compared with bicarbonate, urea and creatinine.

Major Findings: The mean venous plasma acetate concentration of 12 normal human volunteers was 0.025 $\pm$ 0.002 mM (SEM). Prior to the onset of a dialysis treatment the mean venous plasma acetate concentration of 11 chronic dialysis patients was 0.027 $\pm$ 0.002. This was not significantly different from the normal value. The mean arterial plasma acetate concentration in the dialysis patients (prior to dialysis) was 0.048 $\pm$ 0.006. This was significantly higher than the venous concentrations, indicating an arterio-venous difference. This suggests that acetate is used peripherally. Arteriol plasma acetate concentrations were measured in dialysis patients treated with various types of artificial kidneys. At the onset of dialysis, acetate concentrations rapidly rise and usually reach a steady state concentration. At this time net acetate transfer from dialysis to patient [net transfer + QD ($C_{Di}$ - $C_{Do}$)] equals the rate of metabolism. In order to compare rates of metabolism between patients, a metabolic clearance rate (MCR) was calculated:

$$\text{Metabolic Clearance Rate} = \frac{\text{Net transfer (mMol./minute/body weight}}{\text{Arterial plasma acetate}}$$

The metabolic clearance rates for the patients studied range from 6-18 m./minute/kgBW. Some patients achieve relatively high concentrations of arterial plasma acetate ($>$ 5 mM) and this is associated with a low MCR.

Dialysance of acetate has been quantitated and is significantly less than that of bicarbonate and urea.

At the termination of dialysis, acetate concentractions fall in a curvalinear fashion and usually reach baseline values within an hour. When expressed on a log scale, the change in concentration is not linear. This indicates that the metabolism of acetate is not a simple first order process.

In order to compare the rate of acetate metabolism between dialysis patients and normal subjects, eleven normal human volunteers were infused with 4 moles/kg body weight of acetate in one hour. After termination of the acetate infusion, the disappearance of acetate in normal subjects was significantly slower than in the dialysis patients.

Proposed Work:

I. Modify and improve gas liquid chromatography technique by substituting nitrogen carrier gas previously passed over formic acid as the carrier.

II. Metabolic Gas Analysis.

a. Standardization of technique.

b. Metabolic balance studies of $14_C$ acetate during dialyses:

Balanced studies will include measurement of $14_C$ acetate, $14_C$ bicarbonate, $14_{CO_2}$ and plasma levels of $14_C$, during hemodialysis in order to evaluate pathways of acetate metabolism.

III. Acetate Measurement in Dialysis Patients: Investigation of the kinetics of acetate utilization will be continued and acetate levels will be measured during the 4 hours post dialysis using GLC and MGA methods.

IV. Acetate Metabolism in Normal Uremic and Diabetic Patients: To further evaluate the role of acetate in atherogenesis, venous acetate levels will be measured in normal, uremic and diabetic individuals. Acetate infusion studies will be performed in these groups using $14_C$ acetate. The effects of diet, exercise and diurnal variation on plasma acetate will be studied in normal individuals. In addition, glucose and fat tolerance tests will be performed in patients with diabetes mellitus.

Contractor: Mayo Foundation

Amount: $51,535

Title: Uremic Neuropathy

Objectives: To compare peripheral nerve function in patients under short and long hemodialysis sessions. The short dialysis schedule consists of 1-1/2 hours for women and 2 hours for men with a Cordis Dow #5 thrice weekly and the long hemodialysis schedule consists of 3 hours for women and 4 hours for men. Touch measurements and pallesthesia measurements will be compared with clinical symptomatology physical examination and nerve conduction velocities for their sensitivities in detecting differences in neurologic function between dialysis schedules. The same measurements will be conducted in patients undergoing transplantation by studying throughout the pre and post transplant course.

Results: The neurologic disability score, the quantitated test of cutaneous sensation and nerve conduction are neither an early or a reliable criteria to be used for determining adequacy of hemodialysis. Several patients developed the syndrome of uremia on the short hemodialysis schedule without developing clear evidence of worsening nerve function.

Proposed Course: Completed.

Contractor: The Memorial Hospital, Pawtucket, Rhode Island

Amount: $90,000

Title: Anti-Thrombogenic Surfaces: Platelet Interface Reactions

Objectives: To obtain a better understanding of the interaction between blood and artifical surfaces.

Major Findings: Work has been carried out in four separate but interrelated areas. An activity in human plasma that inhibits adhesion of platelets to artificial surfaces has been investigated and characterized. Studies to date indicate that this activity is a lipoprotein or an entity closely associated with lipproteins. Well characterized radiolabeled albumin, fibrinogen, and IgG as well as radiolabeled immunospecific antibodies have been used to devise a radioimmunoassay for use in quantitation of adsorbed plasma protein. In these studies, the amount of antibody bound to adsorbed antigen has been shown to have a linear relationship. An in vitro flow chamber used in the uniform flow mode has been characterized with respect to effects of anticoagulant, effects of flow rate, and effects of use of various normal donors on the adhesion of platelets under flow conditions. Studies to date have indicated that fewer platelets adhere to Cuprophane than to glass or polyvinyl chloride. Studies have been carried out on the growth of human umbilical vein endothelial cells on the inner surfaces of tubes. Endothelial cells were harvested from umbilical veins and allowed to settle within the lumen of a tube. Cells that settled on the inner surface of a tube were allowed to grow for 24 hours under static conditions in tissue culture. Then the tube was placed in flow circuit. It was found that endothelial cells can grow to confluence on the inner surfaces of tubes when flow rates are 1 ml per minute or less. These tubes have an internal diameter of 2 mm.

Proposed Course: Elucidation of the cause of dialysis-induced thrombocytopenia is planned. Continued studies into the growth of endothelial cells in hollow fibers under flow conditions are underway and studies of the effects on blood components by dialyzers *in vivo* is also to be carried out.

Contractor: Minneapolis Medical Research Foundation, Inc.

Amount: $89,628.00

Objective: To assess in vitro mass transfer characteristics of acetate, bicarbonate, pyruvate, lactate, citrate, acetoacetate, and B-hydroxybuterate. Three dialyzers ($1M^2D_4$ Kiil, $1M^2$Gambro-Lundia Nova and 1.3 $M^2$ CDAK-4) were studied. Separate determinations of clearances were made with carbon$^{14}$ radio-labeled sodium salts of each substance added to a blood simulant perfusing the dialyzer in a recirculating system, incorporating a three-liter reservoir.

Another objective was to evaluate the presence of glucose in dialysate to see if it altered intermediary metabolites (acetoacetate and B-hydroxybuterate). Thus, a short term two-way study of five patients (NPO) for ten hours prior to and during the course of dialysis, was undertaken to evaluate the effects of glucose (200 mg/dl) versus no glucose (0 mg/dl) in the dialysate. The study also included a pre- and post-dialysis evaluation of changes in free fatty acids, triglycerides and cholesterol, along with the terminations of serum immunoreactive insulin and glucagon levels. Finally, evaluation of the effects of dialysate concentrations of acetate was continued with seven patients ultimately being evaluated.

Results: Below is illustrated the results of the comparison of the three different dialysis machines with mass transfer characteristics from each of the different molecules.

| | Urea (60)* | $Ac^-$ (82) | $HCO^-_3$ (84) | $Pyr^-$ (88) | $Lac^-$ (90) | $AcAcet^-$ (102) | $BOH\ but^-$ (104) | $Cit^-$ (210) |
|---|---|---|---|---|---|---|---|---|
| $D_3$Kiil | 120 | 88 | 110 | 63 | 72 | 89 | 68 | 43 |
| Gambro | 130 | 95 | 127 | 56 | 79 | 94 | 72 | 48 |
| CDAK-4 | 160 | 98 | 138 | 56 | 78 | 96 | 76 | 44 |

*( ) = mol. wt. in daltons

Dialysis against a glucose containing dialysate caused true plasma glucose to rise during dialysis, while post dialysis, the plasma glucose levels of the glucose vs. non-glucose situation were similar.

The heparin clearing effect on triglycerides was comparable in the two groups with the glyceride levels falling quite profoundly. Cholesterol, as expected, did not respond to heparin administration. While pyruvate and lactate do not show any predictable change across dialysis, relative to the presence or the absence of glucose, the data so far suggests that acetoacetate and B-hydroxybuterate increase much more during dialysis without glucose. These results, are suggestive of gluconeogenesis occurring in a patient dialyzed without glucose in the dialysate.

The heparin clearing effect on triglycerides was comparable in the two groups with the glyceride levels falling quite profoundly. Cholesterol, as expected, did not respond to heparin administration. While pyruvate and lactate do not show any predictable change across dialysis, relative to the presence or the absence of glucose, the data so far suggests that acetoacetate and B-hydroxybuterate increase much more during dialysis without glucose. These results, are suggestive of gluconeogenesis occurring in a patient dialyzed without glucose in the usually resulted in post-dialysis blood pH levels of around 7.50. Probably because of the differing demands for buffering metabolic acid between dialyses, the 30 mg per liter bicarbonate providing complete substitution for a dialysate acetate is inadequate in some patients, causing some of them to become quite acidotic following their longest interdialytic. It is apparent that the EEG % power shows increased slow wave activity pre-to post-dialysis in some patients, even when dialyzed on their standard acetate bath. The removal of acetate from the dialysate solution appeared to have little impact on the deterioration and the EEG activity. The data suggests that the induction of metabolic alkolosis during dialysis patients are driven to a state occasionally of frank alkalosis, even following their longest interdialytic interval, the pre-dialysis % power increases above normal in some and may further worsen if already above normal in others.

Proposed Research: Study completed.

Final Results: Not yet available.

Contractor: University of Minnesota

Amount: $39,992

Title: Fluid Dynamics of Blood Cells and Applications to Hemodialysis

Objectives: The contractor has focused his research in two areas:

(1) Development of a novel blood access
(2) Deposition of formed elements unto filtering surfaces

Major Finding: Work on the blood access device (a self-sealing button attached to segment of prosthetic artery) has proven sufficiently promising in dog tests that plans are being formualted to mass produce and mass test the device prior to human implantation. Work on the deposition of particles on filtering walls has resulted in several noteworthy results.

1. When the duration of a test is properly accounted for, continuous filtration polarization for a diffusible solute theory supplies an upper bound for the lu and platelet data.

2. Wall repulsion effects are apparent but less pronounced than expected from RBC data.

3. There is a surface area exclusion effect by which RBC exclude platelets from a 140 $u^3$ layer around each RBC; the net result of this is a larger concentration of platelets in the RBC depleted layer near the wall than would be expected from the platelet count.

Proposed Course: Work on blood access will be continued by the subcontractor under a separate contract. Work on the deposition of blood particles will be continued using a new technique developed at the University of Minnesota.

Contractor: University of Minnesota

Amount: $100,380

Title: Oxidant Induced Erythrocyte Destruction in Chronic Renal Disease.

Objectives: To define more exactly the frequency of occurrence and nature of oxidant compounds which may occur in dialysis baths. To develop and test techniques for the removal of unwanted oxidant from the dialysis baths.

Major Findings: There are no major findings in this study as it has only been initiated within the last ten months.

Proposed Course: A large population of hemodialysis will be studied to determine the frequency of the previously recorded intrinsic defect in erythrocyte metabolism which causes an increased susceptibility of these red cells to exogenous oxidant stress. If possible, the nature of this defect will be better defined and the relationship between the occurrence of the intrinsic defect and red cell survival in patients dialyzed against both contaminated and oxidant free dialysis baths will be determined. Studies will be performed to test the efficacy of the addition of ascorbic acid to chlorine/chloramine containing dialysis baths as a method for the detoxification of these compounds.

Contractor: University of Missouri School of Medicine

Amount: $39,480

Title: Antinuclear Antibodies in Chronic Dialysis and Renal Transplantation

Objective: To detect the incidence of antibodies to nuclear antigens including fluorescent antinuclear antibodies (FANA). This has been done by screening serum from over 200 patients and followed 50 patients up to 40 months. The influence of age, sex, bloodtype, HLA tissue type, nature of renal disease, time on dialysis, dialyzer reuse, and kidney transplantation on the incidence of antibodies. Studies have been performed to see if particular symptoms, physical findings or laboratory findings can be correlated with the presence of such antibodies.

Results: One or more of three antinuclear antibody types was detected on at least one occasion (titers ranging from 1/10-1/1280) in 46 patients (31%). The number of patients with antibodies to each antigen or combination of antigen were:

| ENA Only | FANA Only | DNA Only | DNA+ ENA | ENA+ FANA | DNA+ FANA | ALL |
|---|---|---|---|---|---|---|
| 16 | 13 | 9 | 4 | 2 | 1 | 1 |

No correlation was seen between antibody titer and age, sex, HLA antigen or blood groups. Coil reuse did not alter the incidence. No correlation was seen with type of disease i.e., pyelonephritis, polycystic kidney disease or glomerulonephritis nor was there an increase in antibody incidence with duration of dialysis use.

Proposed Course: Contract completed.

Contractor: University of Missouri School of Medicine

Amount: $50,175

Objective: The major objective of these studies is to determine the effects of vasodilators added to peritoneal dialysis solution on peritoneal clearances of small and large molecular solutes. Vasoactive effects of vasodilator drugs on the visceral and parietal peritoneal capillary beds should be accomplished with relatively small amounts of drugs added to peritoneal dialysate yielding high local concentrations of the drugs adjacent to and within the peritoneal membrane. Slow absorption of such drugs and obligatory passage of most of that absorbed through the portal venous system and liver should result in minimal concentrations in the systemic circulation.

Results: Clearances of urea, creatinine, phosphate and urate increased significantly with isoproternol, diazoxide and nitroprusside. Nitroprusside demonstrated the largest and most consistent effects with increases in small solute clearances as much as 24% above the control valves.

In three studies with inulin, the clearance of inulin increased from 3 to 4 ml/minute control to 6-9 ml/min. with nitroprusside. The increase in clearances are thought to be related to vasodilitation as well as increase in peritoneal permeability.

Studies to determine mass transfer coefficients with and without nitroprusside have been performed including solute generation rate estimates of body fluid space sizes and frequent sampling during equilibration curves.

MASS TRANSFER COEFFICIENTS (MTC) (ml/min) With and Without Nitroprusside in a Single Patient

| | Urea | | Creatinine | | Inulin | |
|---|---|---|---|---|---|---|
| | MTC | r | MTC | r | MTC | r |
| CONTROL | 22.5 | 0.94 | 8.5 | 0.94 | 1.8 | 0.88 |
| | 23.6 | 0.91 | 9.0 | 0.91 | 1.8 | 0.87 |
| | 21.7 | 0.96 | 7.5 | 0.97 | 1.8 | 0.93 |
| Mean ± SEM | 22.6±0.6 | | 8.3±0.4 | | 1.8±0.02 | |
| NITRO-PRUSSIDE | 30.0 | 0.99 | 9.4 | 0.99 | 2.4 | 0.99 |
| | 36.0 | 0.99 | 14.3 | 0.99 | 4.2 | 0.99 |
| 9 mg/2L | 36.3 | 0.99 | 17.1 | 0.99 | 5.5 | 0.99 |
| | 35.0 | 0.99 | 18.6 | 0.99 | 7.2 | 0.99 |
| Mean ±SEM | 34.3±2.9 | | 14.9±4.0 | | 4.8±2.0 | |
| | $p<0.001$ | | $0<0.025$ | | $p<0.05$ | |

Proposed Work: Longer series of exchanges continuing nitroprusside are being studied to evaluate the prolonged presence of the drug with every exchange. Dose response curves are being determined. Effects on protein losses over a long period of time are being determined. Thiocyanate levels in blood and dialysate are being performed in collaboration with Dr. Popovich.

Contractor: Mt. Sinai Hospital Services

Amount $220,762

Title: A comparison on Androgens for Treatment of Anemia in Chronic Hemodialysis Patients.

Objectives: This study proposes to evaluate four androgen compounds as to their effect on the improvement of anemia as well as their side effects.

Major Findings: There are no major findings in this study as it has just gotten underway this past year.

Proposed Course: Four androgen compounds will be utilized in a randomized crossover study in 100 hemodialysis patients. The four drugs are:

1. Nandrolone Decanoate.
2. Fluoxymesterone.
3. Oxymetholone.
4. Testosterone Enanthate.

These drugs will be given to patients over a six-month treatment period at which time patients will be randomized again and placed into another treatment group for another six months. In addition to the usual history physical and biochemical determinations, special tests of red cell function will be employed. Each patient will have evaluation of red cell 2,3 DPG and whole blood P-50 performed at the end of each control and study period. Red cell mass and plasma volume studies will also be carried out. Extensive questionnaires concerning the side effects of medications used will be completed on each patient at suitable intervals in the study. All data collected will be stored for computer retrieval thereby enabling statistical evaluation of results.

CONTRACTOR: University of Naples

AMOUNT: $34,000

TITLE: Sorbent Studies in the Treatment of Uremia

OBJECTIVE: To develop ingestible adsorbents to sequester urea and other uremic toxicants in the gastrointestinal tract, and to develop with the aid of such compounds suitable adjunctive treatments for patients in chronic renal failure. Also, to explore the feasibility of using oxystarch and related substances as sorbents of uremic wastes to regenerate and recycle artificial kidney dialysate solutions.

MAJOR FINDINGS: The urea adsorbing capacity of oxystarch and of oxycellulose has been increased to a considerable degree by pretreatment with water for about 24 hours. This, together with the observation that an acid pH increases urea binding, caused the investigator to explore the possibility of utilizing the acid gastric environment for urea adsorption in a feasibility study involving two patients. This resulted in the equivalent of a urea clearance of 4.5 ml/m, and based on these considerations a clinical trial with pretreated oxystarch has now been proposed in a group of 10 patients. In addition the Principal Investigator had demonstrated successfully the utilization of activated charcoal for the complete regeneration of dialysis fluid including removal of urea (even though charcoal was known not to adsorb urea). He demonstrated that by cooling the charcoal column the cold carbon will bind unsuspected amounts of urea. Dr. Giordano has built a dialysate regeneration system based on this principle which now permits dialysis with a minimal volume of only 5 liters of dialysate which is being wholly regenerated by a cooled carbon column. The used column can be regenerated completely and repeatedly and quickly by perfusion with warm tap water.

PROPOSED COURSE: Based on the successful demonstration that 24-hour pretreatment with water approximately doubles the urea binding of oxystarch and oxycellulose, a clinical trial with pretreated oxystarch will be undertaken in 10 patients for a period of three months utilizing repeated crossover cycles of oral administration of pretreated and non-pretreated polyaldehydes. Five patients will be maintenance dialysis patients with a GFR of 1 ml/m on a protein intake of 1 g/Kg body weight and five will be patients on conservative low-protein diet treatment with a GFR of 5 ml/m. Chronic periodic hemodialysis utilizing the new cold carbon regeneration of dialysate will be given to two stable maintenance dialysis patients; they will be treated with 4-hour hemodialyses three times weekly utilizing only 4 to 5 liters of dialysate and an activated carbon cartridge containing about 2 kg of carbon which will be regenerated after each treatment period with 10 to 12 liters of tap water at 60°C. The patients' clinical progress will be monitored utilizing a variety of measures.

CONTRACTOR: University of Naples

AMOUNT: $19,500

TITLE: Studies on Peritoneal Dialysis

OBJECTIVE: To continue a study which involves new approaches which promise to increase significantly the efficacy of peritoneal dialysis through use of antidiuretic and vasodilating drugs in the peritoneal lavage solution, or of a combination of peritoneal lavage and the use of oral diuretics in the gut.

MAJOR FINDINGS: During the last 3 years, 3,964 short daily peritoneal dialyses (71.4% at home) have been performed in 18 high-risk patients aged between 8 and 82 years. Six to 10 liters of dialysate have been associated with high-caloric low protein diets (0.6 g/kg in adults). Removal of nitrogen waste products was good and predialysis serum urea averaged $1.30 \pm 0.09$ g/L in 330 determinations. The incidence of peritonitis was 0.18%. Peritonitis was of bacterial origin in 5 instances and of the aseptic type in 2 patients who had been dialyzed for a long time with the same commercial dialysate and the same tubing. The control of anemia, the plasma levels of proteins, albumins, transferrin, C-3c and C-4 levels were satisfactory. But at the beginning of the third dialysis year it became evident that a significant reduction of plasma albumins occurred.

Lipid studies on the patients on short daily dialysis have not shown impaired lipid metabolism, which has been reported in the literature. Cholesterol levels in 8 patients at the start of their dialytic treatment were $187 \pm 11$ mg/dl and $199 \pm 15$ mg/dl under treatment for at least 6 months. The same patients showed trygliceride levels of $117 \pm 12$ mg/dl at the start and $126 \pm 14$ mg/dl under treatment. Furthermore, 4 females aged respectively 40, 47, 54 and 63 years showed that the lipoprotein pattern had not deteriorated under dialytic treatment associated with high caloric diets. From these preliminary data, it is evident that lipid metabolism in patients under peritoneal dialysis must be carefully monitored, and it may be that deranged lipid metabolism in patients under peritoneal dialysis is only the result of the non-homogenous population of patients studied.

PROPOSED COURSE: This project will be continued.

CONTRACTOR: State University of New York, Downstate Medical Center

AMOUNT: $127,146

TITLE: Clinical Efficacy of Sorbents in Uremia

OBJECTIVE: Based on experience with oral administration of oxystarch and activated carbon to patients with advanced renal failure, this study found that the oral administration of activated charcoal will depress the abnormally high blood triglyceride levels of such patients as well as patients on maintenance dialysis. Current studies are testing the hypothesis that oral carbon can depress the hyperlipidemia of dialysis patients to normal levels or below. The importance of the study lies in the fact that about 60% of dialysis patients die because of enhanced atherosclerosis which may be causally related to the abnormally high lipidemia of dialysis patients.

MAJOR FINDINGS: During the past year a clinical trial of oxystarch (35g/day) and activated charcoal (35g/day) administered to four stable patients undergoing maintenance hemodialysis thrice weekly was completed. Following a four-week control period of thrice weekly hemodialysis, subjects were treated for four weeks with both sorbents in divided doses while receiving thrice weekly hemodialysis. After an additional four-week control period, dialysis frequency was reduced to once weekly while oxystarch and charcoal were administered.

Sorbent therapy was well tolerated during thrice weekly dialysis. A slight fall in BUN with no change in serum creatinine, hemogram, or other biochemical and clinical parameters was observed when oxystarch and charcoal were given along with thrice weekly hemodialysis. Oxystarch and charcoal in the doses studied were inefficacious in the trial of reduced dialysis frequency. All four patients deteriorated clinically and two had to be removed from further participation because of uremic pericarditis and profound anemia (each in one patient). One patient developed hyperkalemia and elevated T waves in his EKG. Mean predialysis serum creatinine rose from a control of 19 mg/dl to 28 mg/dl during once weekly dialysis.

Mean serum total cholesterol concentrations diminished significantly ($p<.01$) and reproducibly from 200 mg/dl during control periods to 140 mg/dl after each 4 week trial of activated charcoal/oxystarch; a 23-49% mean fall from control values. Parallel decreases were seen in cholesterol esters (27-42%) and total lipids (24-33%). Hypertryglyceridemia (range 181-543 mg/dl) was corrected in 3 of 4 patients with triglyceride levels $<150$ mg/dl during ingestion of activated charcoal/oxystarch. Triglyceride concentration fell a mean of 27-36% compared to control values. Fat soluble vitamins, free fatty acids and phospholipids were unaffected by the study. Conclusions are that activated charcoal is an effective and safe hypolipidemic agent potentially beneficial to uremic patients.

PROPOSED COURSE: The study will be continued.

Contractor: Northwestern University

Amount: $46,880

Title: Comparison of Maintenance Peritoneal Dialysis and Maintenance Hemodialysis

Objective: To compare peritoneal and hemodialysis based in the following parameters: Mortality, morbidity, biochemical levels, liver function, metabolic bone survey, nerve conduction, velocity and rehabilitation.

Major Findings: Hemodialysis patients were closely matched with peritoneal patients. Each pair was simultaneously entered in the study. Results indicate significant diffences between the two groups. Particularly interesting is the higher hematocrit and the closer to normal EEG encountered in the peritoneal group.

Proposed Course: New patients will be brought into the study. Clinical and neurobehavioral outcome evaluations of the two groups of patients will continue.

CONTRACTOR: University of Pisa

AMOUNT: $20,120

TITLE: Albumin and Hormone Metabolism in Chronic Uremia and Hemodialysis

OBJECTIVE: To elucidate the turnover and biochemical aberrations of albumin, insulin, and growth hormone metabolism in uremia both on and off dialysis.

MAJOR FINDINGS: Studies on insulin metabolism have been carried out in end-stage uremic patients at various periods after the end of hemodialysis, in order to evaluate the acute changes of the hormone turnover induced by the dialytic treatment. On the basis of the results obtained, the following conclusions can be drawn: Patients with chronic, end-stage renal failure are characterized by expanded plasma volume, whence supernormal rates of exit of insulin from the intravascular pool are usually detectable; the interstitial fluid volume is also increased. In these patients, the degradation of the hormone is markedly depressed. The total, plasma-equivalent distribution volume and the exchangeable body mass of insulin are greatly enlarged in uremia as a result of reduced removal, especially by peripheral degradative sites, on the one hand, and of possible concentration gradients through the vascular membrane, on the other. Many patients secrete supernormal amounts of insulin either basally or in response to glucose; also, a consistent proportion of subjects show mild glucose intolerance. No simple relationship seems to exist in the individual patient between these two phenomena, suggesting that sensitivity to insulin may greatly vary in uremia. Dialysis, by removing toxic metabolites, partially restores degradation, further enhances secretion, and definitely improves glucose tolerance.

PROPOSED COURSE: The program proposed for the forthcoming year includes further studies on insulin, human growth hormone (HGH), and triiodothyronine ($T_3$). The studies on insulin will concern three main aspects: 1) insulin resistance in uremia; 2) insulin hypersecretion in uremia; 3) relationships between dialysis and glucose tolerance in uremia.

The kinetic parameters of HGH in the uremic patients will be better defined by increasing the number of experimental determinations of the hormone turnover, including the HGH daytime profile. Furthermore, the pituitary function of uremic patients will be investigated by the following tests: oral glucose, i.v. insulin, l-DOPA, 2-$\alpha$-bromocriptine, arginine, somatostatin, differential protein intake.

As far as the $T_3$ turnover in uremia is concerned, the hormone kinetic data obtained in the patients on conservative management will be compared to those obtained in dialysed patients, both in the pre-dialysis and the post-dialysis state.

Contractor: The Regents of the University of California, San Francisco

Amount: $85,224

Objectives: To characterize those biochemical alterations of the central nervous system, peripheral nerves and skeletal muscle which are present in chronic renal failure and to determine how these alterations are affected by hemodialysis.

Major Findings: Rapid hemodialysis of uremic animals may induce a syndrome characterized by increased cerebrospinal fluid (CSF) pressure, grand mal seizures, and electroencephalographic abnormalities. There is a fall in pH and bicarbonate concentration in CSF, and brain. This syndrome has been called experimental dialysis disequilibrium syndrome. The fall in pH of CSF may be secondary to a fall of intracellular pH (pHi) in brain. Since changes in pHi can alter intracellular osmolality in other tissues, it was decided to investigage brain pHi in uremia, and the effects of hemodialysis. Brain pHi was measured by evaluating the distribution of $^{14}C$-labeled dimethadione in brain relative to CSF. In animals with acute renal failure, brain (cerebral cortex) pHi was 7.06±0.02 (±SE) while that in CSF was 7.31±0.02, both values not different from normal. After rapid hemodialysis (100 min) of uremic animals, plasma creatinine fell from 11.8 to 5.9 mg/dl. Brain pHi was 6.89±0.02 and CSF pH was 7.19±0.02, both values significantly lower than in uremic animals ($P<0.01$), and there was a 12% increase in brain water content. After slow hemodialysis (210 min), brain pHi (7.01±0.02) and pH in CSF (7.27±0.02) were both significantly greater than values observed after rapid hemodialysis ($P<0.01$), and brain water content was normal. None of the above maneuvers had any effect on pHi of skeletal muscle or subcortical white matter.

The data show that rapid hemodialysis of uremic dogs is accompanied by a significant fall in pH of CSF and pHi in cerebral cortex. Accompanying the fall in brain pHi is cerebral edema.

Proposed Course: The principal investigator plans to study changes in brain intracellular water and electrolyte metabolism and energy supplying substrate distribution. In skeletal muscle and peripheral nerve, evaluation will be made of water and electrolyte metabolism, as well as nerve conduction and electromyography studies. Effects of hemodialysis on the aforementioned parameters will then be evaluated, as will changes in the electroencephalagram. It is hoped that such information will elucidate the pathophysiology of dialysis disequilibrium syndrome, peripheral neuropathy and uremic neuromuscular disorders.

Contractor: Southern Research Institute

Amount: $115,884

Title: Activated Carbon Fibers for Use in Artificial Kidney Devices

Objectives: To develop activated carbon fibers suitable for use in artificial kidney absorption devices, and to develop activated carbon hemoperfusion cartridges.

Major Finding: Repeated hemoperfusions were carried out with sheep, goats, a baboon, and uremic patients. Some removal of white cells and proteins were observed in all species, but severe platelet depletion occurred only in the sheep. The activated carbon absorbed heparin and albumin as well as the lower-molecular-weight solutes that diffused through the HEC binder. Less than 1 mg of debris and no toxic substances were removed from cartridges by rinsing with water.

Proposed Course: The major effort this year will be directed toward the selection of a new carbon that will not remove substantial amounts of heparin or protein from blood. The second task will be to improve the cartridge design to decrease the priming volume.

Contractor: Stanford University

Amount: $104,767

Title: Hyperglycemia, Hyperlipidemia and Insulin Resistance in Chronic Renal Failure and in Relation to Hemodialysis.

Objectives: The objective of the study is to define the nature of the abnormalities of carbohydrate and lipid metabolism that occur in patients with chronic renal failure and to evaluate the effect of variations in diet and dialysis on these abnormalities. In parallel studies in animals the role of various circulating factors in the genesis of the hyperglycemia will be studied. The goal of these investigations is to provide understanding of the abnormal carbohydrate and lipid metabolism that occur in patients with chronic renal failure.

Major Findings: The studies completed the last year have focused on three aspects of abnormal carbohydrate and lipid metabolism in uremia. Results indicate that hypertriglyceridemia occurred frequently in chronically uremic man and rat, and that a defect in lipoprotein removal was the major cause of elevated triglycerides. It was also demonstrated that corticoid treatment accentuated the hypertriglyceridemia of chronically uremic rats by exaggerating the removal defect as well as increasing lipoprotein production. In man it was shown that a decrease in dietary carbohydrate intake led to a fall in triglycerides, and this was associated with a decrease in lipoprotein production. Finally, it was shown that insulin resistance in acute uremia could develop in hypophysectonized dogs, suggesting that increased growth hormone secretion is not essential for the development of the insulin resistance of acute uremia.

Proposed Course: The proposed experiments will be divided into clinical studies of patients with chronic renal failure and in biochemical and physiological investigations of animals with acute and chronic renal failure. The clinical studies will look at the dietary effects on glucose and lipid abnormalities in uremia. Dietary manipulations will include decreasing carbohydrate and increasing fat intake on isocaloric diets. Additionally, the polyunsaturated to saturated ratio of fat will be altered and the total cholesterol intake decreased. After such manipulations, as well as other alterations in diets, patients will be evaluated for their fasting and post-trandial levels of glucose, insulin free fatty acids, growth hormone, triglycerides and cholesterol. In addition, it is planned to determine if the presence or absence of glucose in a dialysate fluid effects glucose tolerance in patients with uremia. The animal studies will investigate the circulating causes of insulin resistance and the mechanism of uremic hypertriglyceridemia.

Contractor: University of Utah

Amount: $80,000

Title: Urea Removal System

Objective: Considerable research by various investigators has been devoted to the development of a system to regenerate the dialyzing fluid of the artificial kidney. Activated carbon has been widely used although effectively removing uric acid and creatinine, it does not remove urea in sufficient quantity. The proposed system consists of an asymmetric cellulose acetate or an anion-exchange (positively charged) membrane attached to a secondary loop containing immobilized urease and an ion exchange resin. The membrane will inhibit the transport of the cations such as sodium, calcium, ammonium, potassium and magnesium, but allow urea to permeate through and be converted to ammonium ion. The latter will not be able to back-diffuse and will be removed by the resin. To remove creatinine and uric acid, a column of activated carbon will be placed in the dialysate loop. The advantages will be that ammonium ion is essentially the only cation to be removed in the urea loop.

Proposed Course: This is a new contract.

Contractor: University of Texas

Amount: $121,605

Title: Clinical Development of the Low Dialysis Clearance Hypothesis via Equilibium Peritoneal Dialysis

Objectives: To develop a continuous, ambulatory peritoneal dialysis technique requiring no delivery equipment.

Major Finding:

- A theoretical analysis of the continuous peritoneal treatment was developed The model confirmed the effectiveness of this type of treatment.

- Three patients have been treated with ambulatory techniques with excellent results.

Proposed Course: A total of 10 or more patients will be included in this year's study. Patient outcome will be carefully monitored, and potential complications in treatment identified.

Contractor: University of Washington

Amount: $113,479

Title: Pharmacokinetic Studies in Chronic Renal Disease and Dialysis.

Objectives: This contract is designed to continue the study of drug excretion and metabolism in patients with renal failure in order to predict safe treatment with a number of drugs. These drugs include diazapam and N-acetyl-procainamide, quinidine and clofibrate.

Major Findings: Flucytosine bioavailability and pharmacokinetics have been studied in normal subjects and patients receiving chronic hemodialysis. Nonrenal rates of removal were negligible so that drug elimination in renal failure is severely impaired. Significant quantities are removed by hemodialysis because clearances are similar to creatinine. Using the kinetic data obtained, observed plasma concentration of the drug in hemodialysis patients was close to that predicted. Amikacin: using the kinetic data which was previously obtained in normal subjects and patients with renal failure, stable patients undergoing chronic hemodialysis were given the drug repeatedly and plasma concentrations measured. The observed plasma levels were within 20% of that predicted. Clofibrate: a high pressure liquid chromatographic assay was developed which does not require solvent extraction. Bioavailability studies in normal subjects and hemodialysis patients revealed excellent absorption without significant "first pass" effect. The kinetic data showed slow removal of drug in renal failure and significant removal by hemodialysis due to extensive plasma protein binding. Quinidine: the drug is extensively bound to plasma proteins (albumin and lipoproteins) but binding is not changed by uremia. The nonrenal removal of quinidine in uremic patients is extensive, similar to normal subjects, and is not modified significantly by hemodialysis.

Proposed Course: Continued evaluation of the pharmacokinetic of clofibrate, quinidine, procainamide and its metabolite, N-acetylprocainamide, will be continued in patients with varying degrees of renal failure and those with end stage disease receiving hemodialysis. In particular, work will be carried out to study the metabolism and accummulation kinetics of N-acetylprocainamide. Previous data concerning the pharmacology of procainamide has been questioned because of the finding that its metabolite, N-acetylprocainamide, may have up to 80% of the antiarrythmic activity of the

parent compound. Because of the slower elimination of N-acetylprocainamide, and its lack of metabolism, there is a strong possibility that it may be a more useful antiarrythmic drug than procainamide. Preliminary observations have suggested that this drug is very slowly excreted in renal failure. Therefore, N-acetyl-procainamide kinetics in patients with renal failure and those receiving hemodialysis will be studied during this contract year.

Contractor: University of Washington

Amount: $149,840

Title: Hyperlipidemia and Atherogenesis in Renal Failure.

Objectives: To delineate the mechanism of hyperlipidemia and accelerated atherosclerosis in renal failure patients who are either on hemodialysis or who have undergone renal transplantation.

Major Findings: Previously completed studies in uremic animals indicated that the adaptation to caloric deprivation may be accelerated in renal failure. In addition, completed studies in man and experimental animals suggest impairment and removal of triglyceride-rich lipoprotein from plasma rather then increased hepatic production may be the principal mechanism contributing to hyperlipidemia in uremia. Finally, preliminary studies utilizing tissue culture methodology seem to point to factor or factors present in uremic serum that accelerate the growth of smooth muscle cells.

Proposed Course: The studies to be undertaken fall into two broad general categories:

1. Clinical studies.
2. Studies on the mechanism of atherogenesis utilizing tissue culture techniques.

The clinical studies investigating the mechanism of hyperlipidemia include investigation of the regulation of adipose tissue lipoprotein lipase in renal transplant patients. The low and high density lipoprotein classes will be isolated by ultra-centrifugal techniques from dialysis patients and their apo-proteins identified and characterized by electrophoretic techniques. The studies involving cell culture will look at physiologic and morphologic effects of uremic serum on human arterial smooth muscle cells. Investigations will also be made as to the existence if any, of growth promoting factors in serum from chronic dialysis patients.

Contractor: University of Washington

Amount: $137,428

Title: Quantitation of Response to Erythropoietin of the Anemia of Chronic Renal Disease in Sheep.

Objective: To determine the effectiveness of exogenous erythropoietin in chronic renal failure, a uremic animal model, the subtotally nephrectomized sheet will be employed. The duration of erythropoietin infusion that produces the most effective erythroid response for a given dose in normal sheep will be determined. Then various amounts of purified erythropoietin will be infused into 4 normal, hypertransfused-normal, and uremic sheep. Bone marrow response will be measured by 1) circulating reticulocytes and ferro-kinetic studies, 2) a daily infusion of erythropoietin will be infused into uremic sheep while being maintained on hemodialysis. Fifty-one chromium red cell mass determinations will be used to monitor erythroid stimulation.

Major Findings: Still pending.

Proposed Course: Still pending.

Contractor: University of Washington

Amount: $70,000

Title: Influence of Anions (Acetate or Bicarbonate) on Patient Symptomatology during Dialysis

Objective: This study is to develop additional data on the question of whether standard dialysis could be improved by use of bicarbonate rather than acetate containing dialysate. Particular emphasis is to be on the relative merits of each dialysate with respect to practical clinical problems in the dialysis process such as: incidence of nausea, vomiting, tiredness, confusion, headache; incidence and severity of hypotensive episodes and incidence of angina at varying ultrafiltration rates during conventional dialysis; incidence and severity of hypotensive episodes and incidence of angina during a protocol consisting of ultrafiltration alone followed by dialysis with substantially no ultrafiltration occurring. The symptomatic response of a group of patients subjected successively to large surface area dialysis employing acetate, bicarbonate or a mixture of both anions will be studied. Patient outcome measures will include acetate levels, bicarbonate, $pCO_2$, pH, blood pressure, pulse, continuous performance test, choice reaction time test, short time interval perception test, and presence and degree of a list of symptoms. The ultrafiltration tolerance of a group of hemodialysis patients on large surface area dialysis as a function of the anion composition of the dialysate will be studied. Five dialysate compositions will be utilized: acetate, bicarbonate, a mixture, successive ultrafiltration-dialysis using acetate, and acetate maintaining osmolality constant by addition of urea and dextrose. Each dialysate will be studied nine times on each patient. Patient outcome measures will include acetate levels, bicarbonate, $pCO_2$, pH, osmolality, blood pressure, and pulse.

Proposed Course: This is a new project.

Contractor: University of Washington

Amount: $54,476

Title: Anticoagulant-Bound Hemodialysis

Objectives: Using a heparin bound dialyzer derived from purified heparin, patient trials will be continued. Approximately 10 uremic patient volunteers will be studied by 2-3 experiments each to further characterize performance of grafted Model 4 system using commercially available heparin. Control runs with systemic heparinization will also be run with ungrafted systems for comparison. Isolated high potency heparin will be grafted to dialysis systems to be used in an additional 10 patients for 7 or more hours of dialysis. Each patient in this series will receive 2-3 runs with the grafted system. Systemic heparin control dialysis will be performed on these same patients. In the long term studies the heparinization technique considered best from the above experiments will be evaluated in studies extending over 4-5 months.

Major Findings: In the last ontract period 26 heparin-grafted dialysis systems employing Cordis Dow Model 4 hollow fiber artificial kidneys (HFAK) and heparin coated blood lines have been used successfully in uremic sheep. An additional 8 ungrafted control systems have also been run for purposes of comparison. Results show a significant difference in performance with grafted systems displaying less clotting within the fibers than controls as judged by change in resistance ($\Delta P$) during experiments and by fiber bundle volume loss (FBVL) at the end of each experiment. Heparin from two commercial sources has been grafted to the HFAK. Performance comparison showed a more pronounced nonthrombogenic effect produced by one of these as determined by pressure drop and fiber volume loss, indicating the variable "potency" of heparin acquired from commercial sources. In vitro clearance studies have been performed on heparin grafted dialyzers showing some loss in permeability. Following deacetylation and heparin grafting, urea and Vitamin $B_{12}$ clearances were reduced 4% and 22%, respectively. Six experiments with heparin grafted dialysis systems were carried out in chronic uremic patient volunteers without complications of acute toxicity such as fever, hemolysis and lysis of blood platelets. The average clot free dialysis time achieved in these initial studies was approximately 6 hours.

Proposed Course: This contract will conclude at the end of the current year with further clinical demonstration of the heparin grafted dialyzer system.

Contractor: Vanderbilt University School of Medicine

Amount: $150,243

Title: Neurobehavioral and Clinical Response to Hemodialysis.

Objectives: The aim is to develop objective, quantitative measures of uremic dysfunction of the nervous system in patients with renal failure as indicators of adequacy of dialysis.

Major Findings: In propsective experiments initial dialysis formats favoring "middle molecule" transport tend to normalize the EEG power spectrum regardless of constant or elevated small-molecule (urea) removal, warranting more extensive trials of such differential dialysis. Abnormalities in both the electrophysiologic measures (EEG, visual evoked response) and the cognition-dependent measures (continuous memory test, choice reaction time, continuous performance test) vary directly with the degree of renal failure. As maintenance dialysis is begun and continued, all of these measures appear to improve at different rates to stable levels which seem to be unique to each patient. Results in some of the cognition-dependent measures appear to vary with age and to reveal "learning" or "experience" effect with repetitive test sessions. Conventional amounts of dialysis as measured by urea removal may be greater than required by at least some patients with end stage renal disease for maintenance of normal neurobehavioral function and freedom from clinical uremic symptoms. As such, the measures may be used as objective indicators of adequate dialysis.

Proposed Course: They propose to use neurobehavioral response parameters to determine the effects of, and to optimize the relationships between quantatively measured and controlled metabolite generation and various dialysis formats in order to optimize dialysis treatment. It is hoped that these neurobehavioral test measures will be useful in assessing adequacy of dialysis therapy.

Contractor: Yale University

Amount: $124,981

Title: Carbohydrate Tolerance in Uremia: Insulin Resistance, Hyperglucagonemia and Altered Protein Metabolism.

Objectives: The objectives of this study are 1) to quantitate the degree of overall tissue resistance to insulin in uremia, 2) to define the contribution of increased hepatic glucose release to insulin resistance, 3) to assess the role of hyperglucagonemia and altered tissue response from this to glucagon in the glucose and tolerance of uremia including studies of receptor binding and stimulation of post-receptor intrahepatic processes by glucagon.

Major Findings: Studies have been undertaken to examine 1) the role of the kidney in glucagon metabolism, 2) the effect of uremia on glucagon binding and tissue sensitivity to glucagon and 3) quantitation of insulin resistance in uremia. Mean renal extraction of glucagon in normal animals (415$\pm$ 33 pg/min/g) significantly exceeded filtered load (122 $\pm$ 21 pg/min/g), indicating that peritubular uptake is a major pathway of renal extraction of glucagon. In 70% nephrectomized rats renal extraction of glucagon was negligible despite filtered loads of 204 $\pm$ 47 pg/min/g, indicating that uremia is characterized by a qualitative change in extraction and/or degradation of glucagon by the kidney. The specific binding of 125 I-glucagon by liver cell membranes was increased 120% to 180% in 70% and 90% nephrectomized rats. In these animals adenylate cyclase activity in liver cells in the basal state was increased two to three-fold as was the increase in response to glucagon stimulation. In uremic patients at a constant hyperglycemia ("glucose clamp") glucose utilization and insulin sensitivity were reduced by 50% indicating that resistance to insulin is a major defect in uremia.

Proposed Course: The proposed work will involve investigation in two primary areas of study, each of which will focus on specific aspects of glucose intolerance in uremia. These areas are 1) studies of glucagon binding and tissue sensitivity to glucagon, 2) quantitation and characterization of insulin resistance. Studies utilizing control rats which have undergone 70% and 90% nephrectomy as well as additional rats with a vesico-jugular shunt will be investigated as to the binding of 125 I-glucagon to liver membranes and the effect of addition of glucagon on adenylate cyclase activity. Additional studies will be

carried out to quantify and characterize the insulin resistance seen in uremic states. These include euglycemic insulin clamp studies in uremic patients and patients who are nonuremic but diabetic. Somatostatin infusion studies will be carried out in uremic subjects. Additionally, studies will be carried out to investigate the status of insulin receptors in uremia. These will be done utilizing monocytes from normal subjects and uremic subjects both before and after entry into a chronic dialysis program. Specific binding of 125 I-insulin to these cells will be determined.

Contractor: Zim University Hospital

Amount: $99,750

Title: Comparison of Hemofiltration and Hemodialysis

Objective: To conduct a comparative study of hemofiltration and hemodialysis in an A B A crossover design. A series of outcome measures will be taken over the control and experimental periods with the objective of determining the relative clinical response. Cost data will also be collected so that both clinical efficacy as well as treatment costs can be compared. The study will involve 12-14 patients in an A B A design of 3-6-3 month periods. Extensive outcome measures are to be recorded.

Proposed Course: This is a new project.

ANNUAL REPORT
EPIDEMIOLOGY AND FIELD STUDIES BRANCH

The Chief of the Branch, Dr. P.H. Bennett, was the recipient of the 1977 Lilly Award of the American Diabetes Association for studies of the epidemiology of diabetes mellitus.

Southwestern Field Studies Section

The primary activities of the Southwest Field Studies Section continue to relate primarily to the prospective studies of the natural history of diabetes mellitus and arthritis among the Pima Indians of the Gila River Indian Community.

The availability of long-term prospective information on the population has now enabled estimates of the incidence of diabetes to be made and reveal that the age-adjusted incidence of diabetes mellitus in the Pima population is more than 20 times as high as in Rochester, Minnesota. The incidence is maximal in males between 35 and 44 years of age and in females between 45 and 54 years of age. The incidence of the disease is strongly related to the obesity at baseline with a five-fold range from the least to the most obese groups.

The frequency of obesity in the offspring of normal and diabetic parents has shown that even between the age of 5 and 14 years, at a time when glucose intolerance is extremely rare, the offspring of diabetic parents weigh significantly more than the offspring of two non-diabetic parents. These trends continue in the 15-24 year age range at which time the offspring of two diabetic parents weigh approximately 20% more than those of two non-diabetic parents. These findings suggest that the obesity observed in Pima Indian children and teenagers may, in fact, be part of the diabetes syndrome, and that the propensity to develop obesity may be part of the genetic predisposition to diabetes. Whether or not such changes are present in infants at birth and prior to the development of clinical diabetes in the parents will be investigated from the prospective data which has been collected on all pregnancies occuring on the Gila River Reservation over the past 10 years.

Investigations of the plasma cholesterol and triglyceride concentrations amongst the Pima Indians over a wide spectrum of glucose tolerance have indicated that the levels of these lipids are linearly related to the degree of glucose intolerance. The data indicate that this correlation is, in part, independent of the degree of obesity and that the triglyceride levels correlate with the fasting glucose level, but not with the degree of obesity. Investigation of the distributions of the fasting plasma glucose levels in the Pima Indian population have shown that, like the one- and two-hour post-load glucose levels, they are bimodally distributed. Investigations of the characteristics of the bimodality have indicated that the fasting glucose level may provide a better index of the presence of diabetes than either the one-hour or two-hour post-load levels. The properties of the fasting and two hour glucose levels in combination for the diagnosis of diabetes, and as predictive measures for the development of subsequent diabetes mellitus will be investigated by newly developed techniques of bivariate - bimodal distribution analysis.

The 10 year follow-up study of the Pima Indian population is now almost complete and will provide data concerning the incidence of vascular complications of diabetes mellitus in the population. These observations combined with the post-mortem characteristics of Pima Indians, with and without diabetes mellitus, will provide the most detailed description of the natural history of the vascular complications of diabetes ever performed. It is expected that the data will allow detailed examinations of the prognostic factors which lead to the vascular complications of diabetes as well as providing descriptive data concerning their natural history.

Examination of the glomeruli isolated from the kidneys of diabetic and nondiabetic Pima Indians have been compared with similar data from non-Indian subjects from Cleveland, Ohio. It has been found that the glomerular size, dry weight, and hydroxyproline content of the diabetic glomeruli are increased compared to those of the nondiabetic subjects, but that the glomeruli of normal Pima Indians are larger than those of non-Indian nondiabetics. The hydroxy proline content per mg of dry weight of glomerulus in the two non-diabetic groups is similar.

Studies of the natural history of arthritis and rheumatism in the Gila River Indian Community have focused primarily on the relationships between histocompatibility locus antigens and ankylosing spondylitis, and vertebral hyperostosis. It has been found that the Pima Indians show a less strong association between the presence of ankylosing spondylitis, and HLA-B27 than has been reported among Caucasian populations, suggesting that an alternative HLA type or other immune response gene marker may be implicated in the pathogenesis of ankylosing spondylitis. This hypothesis is being investigated in conjunction with Dr. Dean Mann of the Immunology Branch of NCI to determine if B cell (lymphocyte) antigens more closely related to ankylosing spondylitis in both Pima Indians and Caucasians can be identified.

There have been recent reports that the syndrome of vertebral hyperostosis is also an HLA-B27 associated spondylo-arthropathy. To test this hypothesis, the frequency of HLA-B27 and other HLA A and B series antigens and haplotypes have been examined in the Pima Indians with and without vertebral hyperostosis. In spite of a high population prevalence of vertebral hyperostosis and HLA-B27 antigen, no association between the two could be found. The failure to demonstrate an association in apparent optimal circumstances indicates that vertebral hyperostosis is not a B27 associated spondylo-arthropathy.

## METABOLIC DISEASE EPIDEMIOLOGY UNIT

The search for additional evidence of an association between goiter and the presence of Escherichia coli was continued. No differences between the frequency of Escherichia coli antibodies, demonstrated by quantitative hemagglutination, or between the concentrations of IgA, IgG, and IgM from serum of goiterous and non-goiterous subjects, or in the serum of subjects from endemic and nonendemic areas was found.

| SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space) | U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT** | PROJECT NUMBER Z01 AM 69,000-12 EFS |
|---|---|---|

PERIOD COVERED
July 1, 1976 to September 30, 1977

TITLE OF PROJECT (80 characters or less)
Diabetes Mellitus in the Gila River Indian Community

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

| | | |
|---|---|---|
| PI: | P.H. Bennett, Chief | EFSB, NIAMDD |
| Other: | B. Barclay-White, Visiting Fellow | SFSS, NIAMDD |
| | W.C. Knowler, Staff Associate | SFSS, NIAMDD |
| | P.J. Savage, Staff Physician | SFSS, NIAMDD |
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COOPERATING UNITS (if any)
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LAB/BRANCH
Epidemiology and Field Studies Branch

SECTION
Southwestern Field Studies Section

INSTITUTE AND LOCATION
NIAMDD, NIH, Phoenix, Arizona 85014

| TOTAL MANYEARS: | PROFESSIONAL: | OTHER: |
|---|---|---|
| 8.3 | 3.0 | 5.3 |

CHECK APPROPRIATE BOX(ES)

☒ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☐ (c) NEITHER

☒ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The natural history of the various manifestations and vascular complications of diabetes mellitus are being studied and evaluated in an Amerindian community. Various associated conditions and factors, including heredity, that may have a bearing on the frequency and severity of diabetes and/or its complications are under investigation.

Project Description

Objectives:

1. Determine the prevalence and incidence of diabetes mellitus among those five years of age and over on the Gila River Indian Reservation.

2. Evaluate factors associated with the progression from prediabetic state to subclinical diabetes mellitus and to clinical diabetes.

3. Study risk factors associated with the rate of appearance and progression of vascular complications.

4. Investigate influence of tribe, age, obesity, parity, and other factors on changes in glucose tolerance and their relationship to diabetes mellitus.

Methods Employed:

1. The following tests and examination are repeated at two yearly intervals on all Indians five years of age or more living on the Sacaton Service Unit portion of the Gila River reservation.

   a. Clinical interview
   b. Physical examination
   c. Modified glucose tolerance test and other biochemical tests

   In addition, on those aged 15 years and over:

   d. Fundus photographs
   e. Electrocardiogram
   f. Tests for vibratory sensation threshold
   g. Soft tissue radiographs for vascular calcification

2. Some of the above tests and examinations are also being done on Indians of other tribes or living under different environmental conditions and on non-Indian subjects for comparison.

Major Findings:

1. Prevalence and incidence of diabetes. Among subjects at least one-half Pima and resident on reservation, the age-adjusted prevalence of diabetes (2 hour plasma glucose at least 200 mg/dl) is 21.1% $\pm$ 2.4% (standard error). Prevalence of diabetes is highly dependent on obesity, with age-adjusted prevalence rates of 14.6 $\pm$ 1.5%, 22.8 $\pm$ 1.1%, and 25.3 $\pm$ 1.7% for those with obesity index (weight/height$^2$ in gm/cm$^2$) <2.5, 2.5 to 3.5, and >3.5, respectively.

   The age-adjusted incidence rate of development of new cases of diabetes is 27.6 $\pm$ 2.4 cases/1000 person years at risk, the highest yet reported

and about 21 times the age-adjusted incident rate in residents of Rochester, Minnesota. Diabetes incidence is low in childhood, reaches maxima of 61 cases/1000 person years in males at ages 35-44 and 64 cases/1000 person years in females at ages 45-54. It is strongly influenced by obesity, with age-adjusted incidence rates of 10.7 ± 3.6, 24.5 ± 2.5, and 52.1 ± 6.3 cases/1000 person years in the least to most obese groups, respectively.

Extensive analyses of potential biases in these estimates due to incomplete followup of subjects indicate that such biases are probably small and of little importance.

2. Familial relationships of obesity and hyperglycemia. Although obesity and maturity-onset diabetes are known to be associated, obesity is generally thought to be an independent factor which increases the risk of diabetes. This association has been examined in 1249 Pima Indian children and young adults and their parents. Fathers and mothers were divided into diabetic(d) and nondiabetic(n) on the basis of 2 hr plasma glucose levels (2 hr PG) < or> 200 mg/dl. The percent desirable weights (PDW) and 2 hr PGs (Mean ± SEM) of their offspring are shown:

| Parents | Offspring: Age 5-14 years | | Offspring: Age 15-24 years | |
|---|---|---|---|---|
| | PDW | 2 hr PG | PDW | 2 hr PG |
| NxN | 114±1 | 98±1 | 115±2 | 108±6 |
| NxD | 114±2 | 100±2 | 127±3 | 110±4 |
| DxN | 118±2 | 99±1 | 122±3 | 111±5 |
| DxD | 126±3 | 101±3 | 135±7 | 158±22 |

The offspring of two diabetic parents (genetic prediabetics) were significantly more obese than the offspring of nondiabetic parents ($p < 0.001$), and those with one diabetic parent were intermediate. Since obesity in the genetic prediabetics occurred before abnormalities of glucose tolerance, the results suggest that the development of obesity may be in part an inherited precursor of diabetes.

3. Serum lipids in diabetes mellitus. Fasting plasma cholesterol and triglyceride concentrations were measured in 728 Pima Indians aged 5 years and older whose plasma glucose levels 2 hours after a 75gm oral carbohydrate load ranged from 54mg/100ml to 862mg/100ml. Subjects were divided using 2 hour plasma glucose levels (2 hr PG) into normal (2 hr PG<160mg/100ml, N=352), indeterminate(2 hr PG>160 to 249mg/100ml, N=91) and diabetic(2 hr PG>250mg/100ml, N-225) groups. In those with normal tolerance, both cholesterol and triglyceride levels increased up to the 3rd decade then remained relatively stable with aging. In the normals, cholesterol levels(mean±SEM) were 163±1.7mg/100ml, significantly lower than those in the diabetics, 180±2.6mg/100ml($p<0.001$). Triglyceride levels in the normals were 125±3.3mg/100ml, also lower

than those in the diabetics, 196±8mg/100ml($p<0.001$). The indeterminate group had levels closer to those normals. The triglyceride levels were correlated with fasting glucose ($p<0.001$) but not with degree of obesity. The absence of hypercholesterolemia in both the normal and diabetic Pimas may explain the lower frequency of myocardial infarction previously noted in the Pima.

4. Cluster analysis in the classification of diabetic status. A cluster analysis was applied to a sample of 250 Pima Indians, in whom plasma glucose and insulin were determined fasting, one and two hours after a 75 gm carbohydrate load. A model of a mixture of two bivariate gaussian distributions was used for the joint distributions of the log values of glucose and insulin. The mixture was assumed to consist of a "normal component" and a "diabetic component," each characterized by parameters for means, s.d., and corr. coeff., and a mixture parameter (proportion in the normal component). Maximum likelihood estimates of the parameters were obtained iteratively by a modified Newton-Raphson method. Probabilities of misclassification for normals classified as diabetics and diabetics as normals were estimated. For pairwise combinations of glucose and insulin values probabilities for normals classified as diabetics ranged from 2 to 10 percent, those for diabetics from 7 to 12 percent. These results provide an adequate model to describe the joint distribution of glucose and insulin and objective criteria for classifying diabetic status, with acceptable probabilities of misclassification.

5. Diabetes in Japanese migrants to Hawaii. A modification of the study protocol used in the Pima Indians was applied to immigrants from Hiroshima, Japan to Hawaii. The results will be compared with data from Japanese subjects in Hiroshima and with the Pima Indians to compare factors associated with complications of diabetes in the various areas.

6. Incidence of complications of diabetes. Studies of renal and retinal complications of diabetes and of mortality are being conducted in order to estimate the effects of potentially treatable risk factors, such as hypertension and obesity, on the incidence of these complications. The analyses make use of multivariate techniques for controlling confounding factors.

Significance to Biomedical Research and the Program of the Institute. Because of the unusually long period of follow-up (12 years) in a defined population, we have the opportunity to estimate incidence rates of diabetes and its complications. The development of diabetes has been shown to be largely attributable to obesity. This finding emphasizes the need for further research into the relationships between types of obesity and temporal patterns of weight change with diabetes incidence, studies of which are in progress. The finding of increased obesity in offspring of diabetic parents again emphasizes the interrelationships between diabetes and obesity and is consistent

with the finding of obesity predisposing to diabetes.

Methodology similar to that used in computing diabetes incidence will be used to describe risk factors for vascular complications in diabetics, with a view towards identifying possible preventive measures.

A possible explanation has been given for the previously described low prevalence of coronary heart disease in Pima Indians. While plasma cholesterol and triglycerides are higher in diabetic than in nondiabetic Pimas, cholesterol levels were low in both groups. Thus hypercholesterolemia, an important risk factor for coronary heart disease, is virtually absent in the Pimas.

The separation of subjects into two fairly distinct groups by combination of values of glucose and insulin during a glucose tolerance test supports the concept that diabetes is a distinct entity, rather than part of a continuum with normal glucose metabolism.

Proposed Course:

The long-term follow-up studies of diabetes and its vascular complications will continue. Surveillance of nondiabetic subjects becoming diabetic and of diabetics progressing with their disease gives an opportunity for further study of factors related to diabetes incidence and the development of complications. Specifically, the role of rates of change in obesity and insulin levels in diabetes incidence will be explored, and risk factors for vascular complications will be described.

Publications:

Bennett, P.H., and Miller,M.: Vascular complications of diabetes in American Indians, Japanese and Caucasians, in Baba, S., Goto, Y., and Fukui, I. (eds) Diabetes Mellitus in Asia, Proc. of second symposium, Kyoto, Japan, 1975, International Congress Series No. 390, Amsterdam, Excerptica Medica, 202-207, 1976.

Bennett, P.H., Rushforth, N.B., Miller, M., and LeCompte, P.M.: Epidemiological Studies of Diabetes in the Pima Indians. Recent Progress in Hormone Research 32:333-376, 1976.

Dorf, A., Ballintine, E.J., Bennett, P.H., and Miller, M.: Retinopathy in Pima Indians: Relationship to Glucose Level, Duration of Diabetes, Age at Diagnosis of Diabetes, and Age at Examination in a Population with a High Prevalence of DIabetes Mellitus. Diabetes 25: 554-560, 1976.

Ingelfinger, J.A., Bennett, P.H., Liebow, I.M., and Miller,M.: Coronary Heart Disease in the Pima Indians: Electrocardiographic Findings and Postmortem Evidence of Myocardial Infarction in a Population with a High Prevalence of Diabetes. Diabetes 25:561-565, 1976.

Murphy, R.L., Holford, S.K., Knowler, W.C.: Visual Lung-Sound Characterization by Time-Expanded Wave-Form Analysis. NEJM 296:968-971, 1977.

Savage, P.J., Hamman, R.F., Bartha, G., Dippe, S.E., Miller, M., and Bennett, P.H.: Serum Cholesterol Levels in American (Pima) Indian Children and Adolescents. Pediatrics 58:274-282, 1976.

Fajans, S.S., Bennett, P.H., Freinkel, N., Kipnis, D., Lacy, P.E., Winegard, A.I., (Eds.): Diabetes mellitus. U.S. Department of Health, Education, and Welfare Publication No. (NIH) 76-854. Washington, D.C. U.S. Government Printing Office, 1976, 361 pp.

Hamman, R.F., Bennett, P.H., and Miller, M.: Incidence of diabetes among the Pima Indians. In Geopathology of Diabetes, Advances in Metabolic Disorders, Bennett, P.H., and Miller, M. (Eds.): New York, NY, Academic Press (in press).

## Appendix

Contract #NO1-AM-6-2221 for Pathogenesis of Diabetes Mellitus in the Gila River Indian Community.

Some development of mathematical theory and computation involved in the above project was carried out under this contract with Dr. Harold B. Houser, Department of Biometry, Case Western Reserve University, Cleveland, Ohio. The contract amount is $46,530.00 for the period September 30, 1976 through September 29, 1977. It involves 1.5 person years of effort.

| SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space) | U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT** | PROJECT NUMBER Z01 AM 69,001-08 EFS |
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PERIOD COVERED
July 1, 1976 to September 30, 1977

TITLE OF PROJECT (80 characters or less)

Complications and Outcome of Diabetic and Prediabetic Pregnancies

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

| | | |
|---|---|---|
| PI: | P. H. Bennett, Chief | EFSB, NIAMDD |
| Other: | W.C. Knowler, Staff Associate | SFSS, NIAMDD |
| | P.J. Savage, Staff Physician | SFSS, NIAMDD |
| | B. Barclay-White, Visiting Fellow | SFFS, NIAMDD |

COOPERATING UNITS (if any)

Indian Health Service

LAB/BRANCH
Epidemiology and Field Studies Branch

SECTION
Southwestern Field Studies Section

INSTITUTE AND LOCATION
NIAMDD, NIH, Phoenix, Arizona 85014

| TOTAL MANYEARS: | PROFESSIONAL: | OTHER: |
|---|---|---|
| 1.5 | 0.8 | 0.7 |

CHECK APPROPRIATE BOX(ES)

☒ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

To determine the effects of diabetes mellitus and prediabetes on the complications and outcome of pregnancy in women in the Gila River Indian Community.

The characteristics of all pregnancies among residents of the Gila River Indian Community will be determined. Each pregnant woman will have a modified glucose tolerance test during the third trimester and following delivery and will be retested at approximately two yearly intervals in conjunction with the prospective study of diabetes mellitus in the same community. The characteristics of the pregnancies in women who subsequently become diabetic will be compared to those among women who were diabetic at the time of pregnancy and those who remain nondiabetic.



PHS-6040
(Rev. 10-76)

Project Description:

Objectives: To determine whether pregnancies in prediabetic women are associated with excessive complications during pregnancy or with excessive mortality or morbidity in the offspring.

Methods Employed: The course and outcome of pregnancies in all female residents of the Gila River Indian Community (Sacaton Service Unit area) are followed and recorded in a standardized manner.

All patients in the prenatal clinic of the Sacaton Hospital receive a modified glucose tolerance test during the third trimester of their pregnancy. These patients receive another modified g-ucose tolerance test 24-48 hours post partum and are retested at approximately two yearly intervals to document the development or lack of development of diabetes. Infants will be examined to determine the presence of congenital anomalies and other characteristics, and data related to mortality and morbidity will be compiled. At age five years the children will be reexamined to detect any further abnormalities which may have been inapparent at birth.

Major Findings: Analysis has been deferred pending collection of additional data.

Significance to Biomedical Research and the Program of the Institute: In view of the high prevalence of diabetes in the Pima Indians, studies in the Gila River Community provide a unique opportunity to evaluate the outcome of both prediabetic pregnancies and pregnancies with mild carbohydrate intolerance. Retrospective studies of the course of prediabetic pregnancies have to date produced conflicting results. Although it is well established that patients with severe glucose intolerance, especially those with diabetes of long duration and significant microvascular complications, have an increased prevalence of fetal mortality and maternal morbidity, it is not certain whether treatment will improve the outcome of those pregnancies in which there is mild glucose intolerance. Limited prospective studies of the benefit of treating pregnant women with mild glucose intolerance have so far yielded conflicting results. With the adoption of more stringent criteria for the treatment of mild carbohydrate intolerance in pregnancy it is hoped that this study will be able to add to the evaluation of this problem by monitoring the impact of these therapeutic changes on the outcome of pregnancies on the reservation.

Proposed Course: The pregnancy study will continue in conjunction with the study of the natural history of diabetes mellitus in the Gila River Indian Community. The number of pregnancies recognized to be prediabetic will increase as the mothers studied develop diabetes mellitus.

Publications: None

| SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space) | U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT** | PROJECT NUMBER Z01 AM 69,006-07 EFS |
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PERIOD COVERED
July 1, 1976 to September 30, 1977

TITLE OF PROJECT (80 characters or less)

Gila River Indian Community Autopsy Study

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

| | | |
|---|---|---|
| PI: | P.H. Bennett, Chief | EFSB, NIAMDD |
| Other: | B. Barclay-White, Visiting Fellow | SFSS, NIAMDD |
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Epidemiology and Field Studies Branch

SECTION
Southwestern Field Studies Section

INSTITUTE AND LOCATION
NIAMDD, NIH, Phoenix, Arizona 85014

| TOTAL MANYEARS: | PROFESSIONAL: | OTHER: |
|---|---|---|
| 1.0 | 0.5 | 0.5 |

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The postmortem characteristics of Pima Indians of the Gila River Indian Community are investigated so that findings in subjects with and without diabetes mellitus can be correlated with studies in living subjects.

Postmortem examinations are performed on members of the Gila River Indian Community whenever possible, regardless of whether the subjects have prior evidence of diabetes. Tissues are examined by light and electron microscopy and the histology related to the clinical findings in the same subjects. Trace metal contents of tissues are measured to determine relationships of trace metals to diabetes. The data collection will be continued for several years with periodic analyses of the data.



PHS-6040
(Rev. 10-76)

Project Description:

1. To determine the causes of death, and associated disease processes in members of the Gila River Indian Community being followed in prospective studies on arthritis, diabetes mellitus, cholelithiasis, etc.

2. To ascertain and evaluate the histopathological changes in tissues and cells as they related to diabetes mellitus. To assess their importance and relationship to clinical findings in members of the Gila River Indian Community upon whom autopsies are performed.

3. Evaluate factors and conditions associated with increased or decreased mortality rate in the Gila River Indians.

Major Findings:

1. Renal Pathology: Glomeruli were isolated from the kidneys of 12 diabetic and 15 nondiabetic Pimas and compared with glomeruli from 22 diabetic and 22 nondiabetic non-Indians from Cleveland, Ohio. In both groups of nondiabetics, age did not affect glomerular diameter, dry weight, or hydroxyproline content. Glomeruli from diabetics were larger (diameter), heavier (dry weight), and contained more hydroxyproline per glomerulus and more collagen per mg/dry weight of glomerulus. There was, however, no relationship between glomerular size and content with duration of diabetes. The increased dry weight of glomeruli of diabetics was totally explained by an increase in basement membrane materials. Although nondiabetic Pimas had larger glomeruli than nondiabetic Cleveland subjects, similar effects of diabetes were seen in the glomeruli of Pima and Cleveland subjects.

2. Trace Metals: Chromium (Cr) levels were measured in samples of liver, spleen, pancreas, aorta, kidney, abdominal fat, and head hair of 11 autopsied diabetics and 20 nondiabetics. Analysis was by flameless atomic absorption spectroscopy. Diabetic status was determined by ante-mortem glucose tolerance test or hypoglycemic drug therapy. There were no significant differences between chromium levels in any tissue between diabetics and nondiabetics. There was no consistent direction of the difference between diabetics and nondiabetics in different tissues. Chromium concentrations in head hair, which have previously been reported decreased in diabetics, were not correlated with levels in the other body tissues. The small difference seen in hair chromium levels between diabetics and nondiabetics was due to confounding by age.

Significance to Biomedical Research and the Program of the Institute: As more subjects who have been under long term ante-mortem surveillance are autopsied, more precise clinical pathologic correlations will be available for the study of diabetes mellitus than has previously been possible.

Glomerular basement membrane thickening appears to be responsible for the enlarged glomeruli in kidneys of diabetics. The effects of diabetes

on the kidneys are similar in Pimas and the non-Indian subjects in Cleveland, so that continuing studies of renal pathology in Pima diabetics will be of general applicability.

Depressed levels of tissue chromium in diabetics which were previously reported by others were not confirmed in the Pimas. Head hair chromium levels, which have previously been used in diabetes studies, do not correlate with the chromium levels in other tissues and are age-dependent. Thus, we cannot support the hypothesis that chromium deficiency is a cause of diabetes.

Proposed Course: The systematic collection and cataloging of tissues will continue. As sufficient specimens become available, detailed examinations of various tissues will be made. Further studies of the kidney, including electron microscopy and biochemical quantification of the $\alpha$ and $\beta$ cells of the islets of Langerhans will be the major activity in the coming year.

Publications: None

| SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space) | U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT** | PROJECT NUMBER Z01 AM 69,009-12 EFS |
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PERIOD COVERED
July 1, 1976 to September 30, 1977

TITLE OF PROJECT (80 characters or less)
Natural History of Arthritis and Rheumatism on the Gila River Indian Community

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

| | | |
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| PI: | P.H. Bennett, Chief | EFSB, NIAMDD |
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| | A.M. Spagnola, Staff Associate | SFSS, NIAMDD |

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| TOTAL MANYEARS: | PROFESSIONAL: | OTHER: |
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| 1.5 | 1.0 | 0.5 |

CHECK APPROPRIATE BOX(ES)

☒ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☐ (c) NEITHER

☒ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The development and progression of various forms of arthritis is being investigated in an American Indian population. The patterns of joint disease will be examined to determine if distinctive patterns of degenerative joint disease can be recognized, and if so, their evolution and outcome will be followed. In addition, the prognostic significance of various factors related to rheumatoid arthritis and ankylosing spondylitis will be examined.

The study which includes clinical radiographic and serologic studies of joint disease is part of a prospective study of the Gila River Indian Community, in which studies of diabetes mellitus and related diseases are being undertaken.



PHS-6040

Project Description:

Objectives:

1. Determine the progression of clinical, radiological and serological features of joint diseases affecting the Gila River Indians with particular attention to rheumatoid arthritis, osteoarthritis, ankylosing spondylitis, and gout.

2. Determine environmental and genetic factors associated with development and progression of arthritis and rheumatism.

3. To compare and contrast the clinical course of rheumatoid arthritis in sero-positive individuals with that of sero-negative arthritics.

4. Determine the predictive and prognostic significance of rheumatoid factor in relation to the clinical course of rheumatoid arthritis.

Methods Employed:

1. The following tests and examinations will be repeated on all Indians five years of age and over, living on the Sacaton Service Unit portion of the Gila River Reservation.

   a. Clinical interview
   b. Physical examination
   c. Radiographs of hands, feet, and cervical spine. Males over age 14 and females over age 44 will have a pelvis film taken.
   d. Laboratory determinations: rheumatoid factor, uric acid.

2. The data from the above examinations and tests as well asdemographic, pedigree, environmental information, and data on other conditions and diseases will also be collected.

Major Findings: The mechanism of the association between HLA-B27 and ankylosing spondylitis among the Pima Indians has been investigated. Pelvic radiographs of adult Pima Indians (100 males, 93 females), selected at random, but including an excess with diabetes, were read by 2 observers for evidence of sacroiliitis (SI) according to the New York criteria, without knowledge of age, sex, clinical or HLA status.

HLA-B27 was present in 18% of the Pima population. In those with SI, the antigen occurred in 8 of 16 (50%) males ($p=0.01$) but surprisingly in only 2 of 22 (9%) females. The difference in HLA-B27 frequently between males and females with SI was statistically significant ($p<0.01$).

HLA-B27 was approximately 3 times as frequent in Pimas as in Caucasians. The relationship between SI and B27, weaker than in Caucasians, was present in male Pimas only. The higher population frequency of HLA-B27 accounts only in part for the greater frequency of SI in the Pima. Although, like Caucasians, about 20% of Pimas with B27 have SI, the risk of SI in the B27 negative Pima is much greater than in the Caucasian.

Vertebral ankylosing hyperostosis is a common skeletal disorder among Pima Indians. Prevalence rates of approximately 50% have been reported in Pima Indian males aged 55 or greater. The HLA-B27 antigen is also found more commonly in Pima Indians than in Caucasians (18% vs 6%). Because of the increased prevalence of both vertebral ankylosing hyperostosis and HLA-B27 in Pima Indians, a possible association between the two may exist. Therefore, histocompatibility testing was done on 44 adult male Pima Indians age 55 or greater with classical vertebral ankylosing hyperostosis and 33 age matched controls. No significant association between any histocompatibility antigen phenotype or haplotype and vertebral ankylosing hyperostosis was found. This disease, therefore, cannot be classified with the HLA-B27 association spondyloarthropathies.

Significance to Biomedical Research and the Program of the Institute. The degree of association between HLA-B27 and ankylosing spondylitis in the Pima Indians (and Blacks) compared to Caucasians suggests that there may be other more specific genetic determinants of ankylosing spondylitis which are in linkage disequilibrium with HLA-B27. Well characterized families with B27 negative spondylitis offer the opportunity to search for such determinants utilizing mixed lymphocyte culture techniques.

No evidence was found of any association between vertebral hyperostosis and HLA B series antigens indicating that this disease is not related to the HLA-B27 positive spondyloarthropathies.

Proposed Course: The appropriate relatives of families will be investigated by mixed lymphocyte typing techniques and B-cell typing in an attempt to identify a spondylitis associated antigen which is more specific for both Indians and Caucasians than the HLA-B27.

Publications:

Calin, A., Bennett, P.H., Jupiter, J., and Terasaki, P.: HLA B27 and Sacroiliitis in Pima Indians. J. Rheumatology 4:44-48, 1977.

| SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space) | U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT** | PROJECT NUMBER Z01 AM 69,011-09 EFSB |
|---|---|---|

PERIOD COVERED
July 1, 1976 to September 30, 1977

TITLE OF PROJECT (80 characters or less)

Experimental and Field Studies of Iodine Metabolism and Endemic Goiter

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

| | | | |
|---|---|---|---|
| PI: | Robert L. Vought | Chief, Metabolic Diseases Epidemiology Unit | EFSB NIAMDD |
| OTHER: | Freddie A. Brown | Chemist | EFSB NIAMDD |
| | Arthur L. Williams | Biochemist | EFSB NIAMDD |

COOPERATING UNITS (if any)
Laboratory of Nutrition and Endocrinology, Clinical Endocrinology Branch, NIAMDD

LAB/BRANCH
Epidemiology and Field Studies Branch

SECTION
Metabolic Diseases Epidemiology Unit

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20014

| TOTAL MANYEARS: | PROFESSIONAL: | OTHER: |
|---|---|---|
| 3.25 | 2.25 | 1.0 |

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The search for additional evidence of an association between endemic goiter and the presence of Escherichia coli was continued. This year's findings add additional evidence that goitrous individuals have more antibodies against E. coli than do the nongoitrous. These findings indicate that further research of this relationship can be useful and this course will be continued.

Project Description:

Objectives: The long-term objectives of the program are: (a) to examine closely the principal environmental factors associated with prevalence of goiter and (b) to study the nature of the metabolic defects responsible for the disease. The immediate short-term objectives are to identify and characterize certain microbially-produced goitrogens.

Methods Employed: Development of the quantitative hemagglutination test was continued and approximately 400 tests were done on sera from Greece. Immunoglobulins (A, G and M) were measured also. Production of the E. coli antithyroid substance was interrupted for several months which slowed our pace. That difficulty has now been corrected.

Major Findings: Unfortunately we were unable to demonstrate differences in E. coli antibodies, or between IgA, IgG and IgM serum concentrations between the goitrous and nongoitrous or between endemic and nonendemic areas. Further studies are being carried out.

We are fortunate in obtaining the services of a competent biochemist to work on purification and characterization of the E. coli antithyroid substance. We are hopeful that this can be worked out by the end of the fiscal year.

OFFICE OF SCIENTIFIC AND TECHNICAL REPORTS

Summary

Fiscal Year 1976

The Office of Scientific and Technical Reports (OSTR) prepares and disseminates the bulk of the regular and special program reports required of the Institute by the Congress, the DHEW and the NIH. Associated functions include the preparation of staff papers, assisting in the preparation of professional publications and Congressional reports, and responding to inquiries of all types.

Congressional mandates in arthritis, diabetes and digestive diseases in recent years have significantly increased the Institute's varied and complex activities, and reporting on all of these activities has severely taxed the comparatively small OSTR office staff. For example, one staffer devoted one month in FY 1976 solely to answering inquiries about the recommendations of the National Commission on Diabetes.

Scientific and Technical Reports:

During FY 1976 OSTR staff members prepared comprehensive special reports on seven different research areas in association with Congressional appropriation hearings. These included exhaustive reports on the state of the art in arthritis, diabetes, digestive diseases, kidney disease, cystic fibrosis, nutrition and Cooley's anemia. Other related reports included the voluminous budget narrative, which focuses on intramural research, and the Director's opening statement, which highlights selected intramural and extramural findings. Certain special reports, such as one on diabetes, which involve a number of Institutes, call for input from these Institutes and coordination with fellow information staff members. All reports

require a concerted effort by OSTR staffers, with regard to report content and an often tight deadline.

The weekly reports are brief summaries written in lay terminology about significant findings reported in the literature by NIAMDD scientists and grantees. Prepared by the OSTR's science writer, these reports reflect the entire scope of the Institute's research interests and programs, and are used primarily as a basis for the various special reports and related material required of the NIAMDD by the Congress, the DHEW, and the NIH. The weekly reports also are distributed routinely to various Institute staff members, News & Features from NIH, the NIH Record, and other interested parties. They also are distributed upon request to representatives of the various news media, and to the general public.

The following are typical of the weekly reports prepared during FY 1976: (1) identification of 51 cases of "Lyme arthritis," a possibly new, infectious form of mild arthritis, in the Connecticut River valley; (2) ear acupuncture for obesity, as well as treatment with human chorionic gonadotropin, not associated with any significant weight loss; (3) cimetidine markedly inhibits gastric acid secretion in duodenal ulcer patients without affecting gastric emptying, or pancreatic and biliary function; (4) reversal of hyperglycemia and glucosuria in diabetic rats by a single fetal pancreas transplant (previously cultured in a normal rat), suggesting that good blood sugar control in the host may enhance the function of the grafted organ, and (5) large doses of antithymocyte globulin significantly improves the 12-month functional graft survival rate in patients undergoing kidney transplantation.

Press Activities:

Press and related activities form an integral part of the OSTR's overall public information program. In line with established policy to publicize the NIAMDD's research activities and programs, the OSTR prepares and disseminates a representative number of press releases each fiscal year. The same are distributed widely to science writers on various metropolitan newspapers, radio and television newscasters, a great many scientific journals, such as the Journal of the AMA, and other outlets.

Representative press releases in FY 1976 included, for example: (1) initiation of the first cooperative clinical trial between the United States and the Soviet Union in the field of arthritis; (2) establishment of a number of Diabetes Research and Training Centers to increase the scope and tempo of Federally-supported research and training activities on this disorder; (3) appointment of 17 members to the National Commission on Digestive Diseases; (4) awarding of a $290,000 contract to the Cystic Fibrosis Foundation for an in-depth study of this incurable genetic disease, and (5) the appointment of 12 public members of the newly created National Diabetes Advisory Board.

On May 9, 1977 U.S. News & World Report featured a lengthy interview with Dr. Harold P. Roth on the subject of ulcers. During the course of this interview Dr. Roth mentioned the drug cimetidine, which he called "a drug that has more promise than anything we've had up to date." This reference resulted in a spate of inquiries directed to the OSTR from news media representatives, Capitol Hill, the general public, and ulcer victims and their friends and relatives. Most inquiries centered upon the availability of cimetidine. OSTR staff members provided the information that cimetidine is still an experimental drug, but that its manufacturer, Smith,

Kline & French, plans to market it under the trade name of Tagomet within six months to one year.

An often overlooked function of the OSTR is servicing the NIH house organ, the NIH Record. During FY 1976 OSTR staff members prepared an average of three stories per month exclusively for this publication. In addition, the NIH Record routinely received copies of all NIAMDD press releases, which it often uses, as well as copies of all NIAMDD weekly reports. Representative NIH Record stories in FY 1976 included (1) Dr. Salans' appointment as Associate Director; (2) revising and updating of "Exchange Lists for Meal Planning;" (3) the Tenth Annual Research Contractors Conference; (4) Robert L. Campbell's retirement; (5) science writers seminar on endocrinology, and (6) naming of Drs. Neufeld and Tabor to the National Academy of Sciences.

Public Inquiries:

Inquiries from the general public deal with such diverse subjects as specific disease areas, controversial forms of therapy, so-called "breakthroughs" in particular research areas, financial aid from the Federal Government, and "cures" for specific diseases. Such inquiries may originate with ill individuals, their relatives and friends, students engaged in science projects, physicians and scientists. Considerable staff time is devoted to answering telephone and letter inquiries, and, if referral to another agency is indicated, this is done with dispatch.

OSTR staff members, in FY 1976, replied to 1,794 telephone inquiries and 1,770 letter inquiries, a total of more than 3,500 such inquiries, exclusive of routine requests for publications.

Congressional Inquiries:

Inquiries from the Congress (and the White House) are many and varied, and are ever increasing in number. Such inquiries run in spurts and apparently can be motivated by any number of factors. For example, in FY 1976 monthly inquiries ranged from a low of 20 in December 1976 to a high of 55 in March 1977. The latter spurt reflected nationwide interest in the recommendations of the National Commission on Diabetes.

Congressional inquiries invariably have tight deadlines and, thus, are given priority over every other OSTR function. All are answered promptly and efficiently and the gist of each response is preserved and filed. Most inquiries concern ill constituents and their friends and relatives, funding of individual grants and specific research programs, proposed "cures" for various ailments, and requests from constituents for financial assistance.

Since 1963 Congressional inquiries directed to the OSTR have skyrocketed from one or two monthly to the present average of 27 per month. During FY 1976 OSTR staffers replied to 329 Congressional inquiries.

Publications:

The May 1977 issue of the Ladies Home Journal featured an article entitled "Eat and Grow Beautiful," together with the notation that the NIAMDD publication - Facts About Nutrition - is available from the OSTR. This resulted in a veritable avalanche of requests from every state in the union, as well as all the provinces of Canada, and a backlog of FAN requests that continues to grow. Despite the fact that all OSTR staff members have taken turns at filling these requests, and several have logged more than a few hours of overtime in this regard, the requests continue to accumulate. To date (in one month's time), the OSTR has mailed out approximately 5,000

copies of FAN.

Several new publications prepared by the OSTR in FY 1976 have proven to be enormously popular - Facts About Obesity and How to Cope with Diabetes. The former is in booklet form while the latter is, at present, being distributed as a fact sheet. It is "in press," however, and its appearance in booklet form is expected momentarily. In line with a recent Congressional mandate to educate the public about diabetes, the OSTR also has revised and updated a brochure entitled Foot Care for the Diabetic, which currently is being distributed as a fact sheet. This publication, too, will be converted to booklet form in the near future.

Radio and Television:

Several radio and television spot announcements released by the NIH Office of Information concerning specific NIAMDD publications have evoked a constant stream of requests for copies of these publications. Thus, an area high school student is employed part-time by the OSTR to fulfill these requests. Despite a determined effort by this dedicated employee, however, a substantial backlog of such requests continues to accumulate.

During FY 1976 OSTR staff members coordinated a number of radio and television appearances by NIAMDD scientists and grantees. For example, Dr. Denis McCarthy addressed the subject of cimetidine for peptic ulcers on a Channel 9 newscast in the Washington, D.C. area, and Dr. Harold Roth discussed peptic ulcer therapy on a Miami, Florida television outlet. In New York City an OSTR staffer personally arranged for appearances by NIAMDD grantee Dr. Israeli Jaffe on CBS radio and NBC television concerning the multi-center controlled trial of penicillamine in rheumatoid arthritis.

Exhibits:

The OSTR also conducts the NIAMDD program of scientific exhibits used

primarily in professional education. Still operating under severe travel and exhibit restrictions, the Institute was able to exhibit at only seven medical and scientific meetings around the country, which were viewed by approximately 40,000 physicians, scientists and paramedical people. Institute exhibits on the artificial kidney, human growth hormone, cystic fibrosis and general research, both intramural and extramural, were shown at these meetings. Various publications and reprints are usually distributed by the OSTR staff member who mans these exhibits. A new exhibit on diabetes among the Pima Indians and a smaller portable exhibit on diabetes are currently being developed.

Science Writers' Seminars

The OSTR, in collaboration with The Endocrine Society and with the logistical support of the Scientific Communications Section, organized a Science Writers' Seminar on Endocrinology to acquaint writers with progress in endocrinology. Summaries of talks along with biographical sketches of participants were prepared for the two-day meeting, March 14-15, at the National Institutes of Health. A great deal of press coverage and publicity was obtained from the discussions on hormonal function in the male, hormones and behavior, hormones and reproduction, and hormones and cancer.

The OSTR also participated in an NIH Seminar for Science Writers on Research on the Gastrointestinal System. Press summaries were prepared on papers delivered by NIAMDD scientists concerning the current status of the treatment of acute liver failure, and on gastrointestinal hormones.

# OFFICE OF THE DIRECTOR

Summary

Fiscal Year 1977

Dr. G. Donald Whedon, Director
Mr. F. L. Mills, Executive Officer

The Office of the Director has overall responsibility for directing and coordinating basic laboratory research; clinical investigations; extramural activities such as research grants, research fellowships, and training grants; contract research and developmental programs; and epidemiologic and clinical field studies. In addition, this office cooperates with other scientific organizations in coordinating medical research in the Institute's fields of interest; cooperates and maintains liaison with other Institutes and staff constituents of other Federal agencies; and participates in determining policy governing the National Institutes of Health. It is also responsible for the collection and dissemination of informational material to interested professional and lay individuals and groups.

During 1977 legislative actions once again directly affected the operations of the Institute. The "Arthritis, Diabetes and Digestive Disease Amendments of 1976", PL 94-562 were enacted October 19, 1976. These amendments created the National Arthritis Advisory Board, the National Diabetes Advisory Board and the National Commission on Digestive Diseases.

The Boards have been established to review, evaluate and implement the National Arthritis Plan and the National Diabetes Plan as developed by the National Commission on Arthritis pursuant to the National Arthritis Act of 1974 and the National Commission on Diabetes pursuant to the National Diabetes Mellitus Research and Education Act. The Institute has obtained space for the two Boards in the Federal Building in Bethesda and is coordinating efforts to supply the Boards with necessary staffing and other support services.

PL 94-562 also created the National Commission on Digestive Diseases which is charged with the responsibility of developing a long-range plan for the use and organization of national resources to deal effectively with digestive diseases. As with previous commissions, the Institute serves as the chief administrative support for the Commission throughout its projected 18 month life-span. In that capacity the Institute will supply both staff and research support for travel, personnel, committee management, legislative liaison, etc.

Several important changes in personnel occurred during FY77. Both the Executive Officer, Mr. Frank L. Mills, and the Personnel Officer, Mrs. June D. Ardizzone, retired. While replacements are being recruited, Mrs. Constance L. Bishop is serving as Acting Executive Officer and Ms. Mettah Kollmann is serving as Acting Personnel Officer. In the Intramural Research Program, Dr. Robert M. Friedman was appointed Chief, Laboratory of Experimental Pathology, and Dr. Paul Berk was appointed Acting Chief of the Digestive Diseases Branch.

Dr. Lester B. Salans is Chief and Dr. Samuel W. Cushman is Associate Chief of the new Section on Cellular Metabolism and Obesity in the Epidemiology and Field Studies Branch. Dr. Arnold Brossi is the new Chief of the Section on Medicinal Chemistry in the Laboratory of Chemistry.

The US-USSR Program continued throughout FY77 with individual visits by Soviet scientists to American research institutions participating in the program and with the US-USSR Cooperative Program on Arthritis meeting in San Francisco, California, held June 23-24, 1977. At this meeting problem area no. 1 "Evaluation of treatment and functional assessment of rheumatoid arthritis (RA) and other connective tissue diseases" and problem area no. 2, "Microbiological and immunological investigations in RA and SLE" were reviewed and plans made for future mutual research. The Institute continues its role as U.S. leader for the cooperative program with research and administrative support contracts supporting the US-USSR Cooperative Program. In addition, Institute staff continued their central role as liaison between the Soviet and American participants.

The Contract mechanism continues as an important tool for research and administrative support of the Institute. Obligations under the contract mechanism during FY '76 totaled $12,046,000. In FY '77, a similar or greater volume is anticipated; as of July 1, 109 contracts were in effect.

Troublesome staffing problems encountered during the previous fiscal year have been largely resolved except for the low average grade level which hampers recruitment and retention of qualified personnel. The workload generated by the emphasis being placed on administrative close-out of about 100 expired contracts has been heavy. In addition, the number of reports required by the Secretary/DHEW, PHS, and DCG, etc., has rapidly escalated. New and revised procurement regulations have reached unprecedented proportions.

Great emphasis by the Department is being placed on increasing the percentage of competitive awards by reducing non-competitive procurements to the absolute minimum. Early project planning is being stressed to achieve a more even distribution of contracting activity throughout the appropriation year. Several evaluations of research priorities/state-of-the-art procurements are underway.

The activities of the Office of Scientific and Technical Reports are included in a separate report which precedes this summary.

In the area of employee training, four courses were offered during 1976-77. Two courses in chemistry (Basis Ideas in Chemistry, I, and Advanced Ideas in Chemistry, VI) continued to be popular and successful. Course work consisted of both classroom and laboratory training and stressed understanding of the concepts underlying biochemical research. A continuation of Basic Mathematics, a course begun in 1975-76, offered a review of arithmetic (number systems, basic operations, fractions), charts and graphs, the metric system, exponents, etc. These three courses are designed for employees who have little or no formal training in the sciences and emphasize a practical rather than theoretical approach. Upon completion of the training, supervisors are

encouraged to utilize their employee's newly acquired skills. The Institute will continue to offer the introductory chemistry and math classes in the fall of 1977 and hopes to offer a continuation of advanced chemistry (VII).

A fourth course, offering basic training in tissue culture, was begun in February of 1977. This introductory course in the basic principles and methods of in vitro cultivation of mammalian cells is designed for individuals with little or no experience in tissue culture techniques. Lectures and laboratory sessions and demonstrations cover such topics as primary cultures, characterization and maintenance of normal and transformed cells, cell nutrition and growth, differentiated function in vitro, cloning, cell hybridization, karyotyping, sterility, and quality control.

During the year, short-term training opportunities have been arranged for 135 secretarial-clerical employees, 31 supervisory managerial personnel and 133 members of the professional staff, as part of this Institute's continuing commitment to the training of its staff to the fullest possible extent. These numbers are not completely indicative of Institute support, however, since they do not include the fourth quarter training supported by NIAMDD.

Equal Employment Opportunity

J. Harrison Ager continues to serve the Institute as EEO Specialist and Counselor. In this capacity he attended the Fifth Annual Xavier-DRR Minorities Biomedical Science Symposium in New Orleans, the bi-monthly NIH EEO Advisory Council meetings and serves as that committee's representative on the Review Committee to Study Employee Conflict. Mr. Ager also counsels NIH employees on EEO concerns, assists the Peer Promotion Review Committee, publishes the Institute news letters and gathers research journals for distribution to minority college research programs throughout the U.S.

Mrs. Linnie Sloan is in her third year as EEO Counselor for the Institute. She has handled numerous EEO complaints both within the Institute and for employees in other BID's. She participates in formulating recommendations to the Director, NIH, for implementing EEO Affirmative Action goals and is serving as the Chairman of the Organization Committee, EEO Council, as well as of the Select Committee advising the Director, NIH, on current concerns of the EEO community.

The Annual Institute EEO Meeting was held May 17. At that meeting Mr. Edward Nicholas, Director of Personnel Management, NIH, made a presentation of the Factor Evaluation System (FES), and the Institute Director presented two EEO Special Achievement Awards to employees of the Institute. The laboratories and branches also held their own individual EEO meetings throughout the year. During the period April 24-30 the Institute actively participated in the NIH Secretaries Week. As part of the effort, NIAMDD sponsored a seminar by Ms. Anne L. Haulsee, Consultant, Women's Programs, Career Empowerment for Women.

The NIAMDD EEO Advisory Committee continues to meet monthly to discuss issues and concerns and to formulate recommendations to meet EEO objectives as spelled out in the Institute's Affirmative Action Plan. The Advisory Committee made a survey of concerns and problems that face visiting scientists and guests workers at NIH and will make recommendations for improvement regarding these concerns and problems.

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